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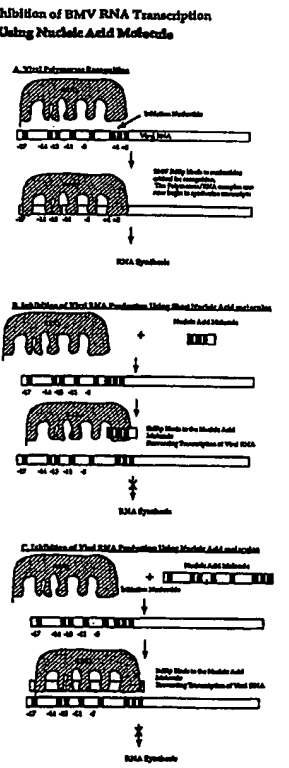
(54) Title: USE OF NUCLEIC ACID MOLECULES AS ANTIVIRAL AGENTS

(57) Abstract

A linear single stranded nucleic acid molecule capable of specifically binding to a viral polymerase and inhibiting the activity of said viral polymerase.



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DESCRIPTION

Use Of Nucleic Acid Molecules As Antiviral Agents

Background of the Invention

Viruses are obligate parasites that depend upon the infected host for many of the basic processes needed for a successful infection. Because viruses depend on the enzymatic and synthetic functions of the host cell, it is very difficult to treat viral infections without affecting cellular processes. Given that several viral diseases are at pandemic proportions, including influenza, AIDS, and hepatitis, the design of effective virus-specific drugs is increasingly important.

For example, potential antiviral drugs are screened to determine whether the drug can preferentially inhibit a viral process. One such drug is Aziduovir, which is utilized more readily by the reverse transcriptase of the human immunodeficiency virus (HIV) than by host cellular polymerases. However, Aziduovir and other viral inhibitors were generally discovered through an intensive and costly drug screening program.

Other antiviral treatments, including interferon, cause significant and widespread changes in the cell and hence lead to a number of side effects, including fever, nausea, and other discomfort.

Viral nucleic acid (RNA/DNA) replication, a process fundamental to viral pathogenicity, requires specific recognition of the nucleic acid features by proteins. RNA-dependent RNA polymerase (RdRp) is a complex composed of viral and cellular proteins that directs viral RNA synthesis from infecting RNA templates. Many viral RdRp proteins have been sequenced and analyzed. However, a comprehensive mechanism describing RNA synthesis is lacking. Consequently, general knowledge of RdRp is significantly less than that of other RNA and DNA polymerases.

Several approaches have been developed for the inhibition of viral protein function using nucleic acid based techniques. A number of these techniques are described in Gold *et al.*, 1995, *Annu. Rev. Biochem.* 64, 763-97; incorporated herein by reference in its entirety. For example, antisense molecules and ribozymes, have been successfully utilized to prevent the translation of mRNA into protein. Alternatively, oligonucleotides have been found which are able to bind directly to protein and thereby reduce or eliminate its function.

One approach for discovering protein binding oligonucleotides is through a process known as *in vitro* selection. From a random pool of RNA molecules, sequences are identified which are capable of binding to specific proteins though cycles of affinity selection. For example, Tuerk *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89, 6988-6992 describes the use of the selection procedure to identify a ligand capable of binding to HIV reverse transcriptase and inhibiting cDNA synthesis. Other nucleic acid ligands (aptamers) have also been described that bind to proteins with high affinity. However, all these RNA aptamers are highly structured with complex secondary and tertiary structures.

Thus, there is a continuing need for agents that specifically inhibit viral replication.

Summary of the Invention

The invention provides nucleic acid molecules that are useful as specific inhibitors of the viral polymerase. In particular, the present invention provides an oligonucleotide of at least four nucleotides, wherein the oligonucleotide comprises a viral nucleic acid sequence which includes the viral initiation nucleotide. Preferably, the oligonucleotide includes viral promoter and initiation sequences.

In a preferred embodiment, the invention features a linear single stranded nucleic acid molecule capable of

binding to a viral polymerase thereby inhibiting the polymerase activity and as a result viral replication is inhibited. These nucleic acid molecules may include preferably initiation sequences, or other sequences necessary for the initiation of, for example, RNA transcription by a viral RNA polymerase. The nucleic acid molecule preferably includes the initiation nucleotide and at least the first, and more preferably the first two nucleotides which are 3' of the initiation nucleotide in the viral sequence.

By "nucleic acid molecule" is meant a macromolecule comprised of at least one nucleotide. The nucleic acid molecule of the invention may be DNA, RNA or a mixture thereof. Preferably, the nucleic acid molecule is comprised of DNA. More specifically, the nucleic acid molecule of the invention comprises nucleotides with modifications in the base, sugar or phosphate groups. It is also preferred that the nucleic acid molecule of the invention comprise at least about four to at least about fifty, more preferably at least about forty, and even more preferably at least about twenty to at least about thirty five, specifically 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 contiguous nucleotides, and that the nucleic acid molecules preferably have identity to the wild-type viral sequence from which they are derived. The nucleic acid molecule may optionally also include cap structures at the 5′ or 3′ end.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see for example, Usman and McSwiggen, supra; Eckstein et

al., International PCT Publication No. WO 92/07065; Usman et al., International PCT Publication No. WO 93/15187; Uhlman & Peyman, supra) all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art and has recently been summarized by Limbach et al., 1994, Nucleic Acids Res. 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acids include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl¥, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, and others (Burgin et al., 1996, Biochemistry, 35, 14090; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used at any position, for example, within the catalytic core of an enzymatic nucleic acid molecule and/or in the substrate-binding regions of the nucleic acid molecule.

By "unmodified nucleotide" is meant a nucleotide with one of the bases adenine, cytosine, guanine, thymine, uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleotide" is meant a nucleotide which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

By "cap structure" is meant chemical modifications which have been incorporated at the terminus of the oligonucleotide. These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell.

By "oligonucleotide" as used herein, is meant a molecule comprising two or more nucleotides.

In another preferred embodiment, the nucleic acid molecule of the invention, a plurality thereof, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed below are useful to prepare an antiviral composition. The antiviral compositions of the invention are particularly useful in methods to inhibit the infection or replication of viruses which are able to use the host's cellular machinery to generate viral polymerases. A non-limiting example of such a virus are (+) single strand RNA viruses, including the alpha virus super-family, which includes bacterial, plant and animal viruses. Other examples of viruses which may be inhibited by the nucleic acid molecules of the present invention include but are not limited to hepatitis C virus, Hepatitis B, Hepatitis A, HIV,

Also provided is a method to inhibit or treat a viral infection that employs at least one nucleic acid molecule of the invention. The method comprises the administration to a cell having, or suspected of having, a viral infection, an effective amount of at least one nucleic acid molecule of the invention, wherein the nucleic acid molecule comprises the initiation nucleotide for the virus.

By "effective amount" is meant, the minimum quantity of the nucleic acid molecule necessary to induce a reduction in the viral load carried in an cell, tissue or whole organism.

In another preferred embodiment the nucleic acid molecule includes modifications selected from a group comprising 2'-O-alkyl (e.g. 2'-O-allyl; Sproat et al., supra); 2'-O-alkylthioalkyl (e.g. 2'-O-methylthiomethyl; Karpeisky et al., 1998, Nucleosides & Nucleotides 16, 955-958); L-nucleotides (Tazawa et al., 1970, Biochemistry 3499; Ashley, 1992, J. Am. Chem. Soc. 114, 9731; Klubmann et al., 1996, Nature Biotech 14, 1112); 2'-C-alkyl (Beigelman et al., 1995,J. Biol. Chem. 270, 25702); 1-5-Anhydrohexitol; 2,6-diaminopurine (Strobel et al., 1994, Biochem. 33, 13824-13835); 2'-(N-alanyl) amino-2'-deoxynucleotide; 2'-(N-beta-

alanyl) amino; 2'-deoxy-2'-(lysyl) amino; 2'-O-amino (Karpeisky et al., 1995, Tetrahedron Lett. 39, 1131); 2'-deoxy-2'-(N-histidyl) amino; 5-methyl (Strobel, supra); 2'-(N-b-carboxamidine-beta-alanyl) amino; 2'-deoxy-2'-(N-beta-alanyl) (Matulic-Adamic et al., 1995, Bioorg. & Med. Chem. Lett. 5,2721-2724); xylofuranosyl (Rosemeyer et al., 1991, Helvetica Chem. Acta, 74, 748; Seela et al., 1994, Helvetica Chem. Acta, 77, 883; Seela et al., 1996, Helvetica Chem. Acta, 79, 1451).

In yet another preferred embodiment the 5' cap structure is selected from a group comprising inverted abasic residue,. 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details see Beigelman et al., International PCT publication No. WO 97/26270, incorporated by reference herein).

In yet another preferred embodiment the 3' cap structure is selected from a group comprising, 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide;

3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moeity; 5'-5'-inverted abasic moeity; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moeities (for more details see Beaucage and Iyer, 1993, Tetrahedron 49, 1925; incorporated by reference herein).

In another preferred embodiment, the nucleic acid molecule of the present invention is conjugated with another moiety including but not limited to abasic nucleotides, polyether, polyamine, polyamides, peptides, carbohydrates, lipid, or polyhydrocarbon compounds. Those skilled in the art will recognize that these molecules may be linked to one or more of any nucleotides comprising the nucleic acid molecule at several positions on the sugar, base or phosphate group.

In another aspect of the invention, the nucleic acid molecules that bind to viral polymerases are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the ribozymes are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of ribozymes. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecules can bind specifically to viral polymerase. Delivery of nucleic acid molecule expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for

introduction into the desired target cell (for a review see Couture and Stinchcomb, 1996, TIG., 12, 510). In another aspect of the invention, nucleic acid molecules that bind to viral RNA polymerases and inhibit viral replication are expressed from transcription units inserted into DNA, RNA, or viral vectors. By "patient" is meant an organism which is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which enzymatic nucleic acid molecules can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

The present invention may be superior to other known antiviral agents because it requires only the nucleic acid sequence(s) for initiation of viral nucleic acid synthesis. Thus, the sequence is readily prepared, does not require complex manipulation by molecular biology techniques, and only a minimal screening regime is necessary. Moreover, the present oligonucleotides may be modified to increase their efficacy at lower concentrations, e.g., modified so as to contain nucleotide analogs or circularized, for RNAs, to provide agents that are more stable in vivo so as to, for example, have increased resistance to degradation in serum. the chemistry of the nucleic acid molecule may also be altered to increase the binding affinity of said nucleic acid molecule. This may be effective in reducing the effective amount of nucleic acid molecules. Further, given that every virus will have a minimum of one sequence needed for the initiation of nucleic acid synthesis, at least one therapeutic can be designed for every virus. In addition, these inhibitors can also be used to disrupt other steps in viral replication, e.g., translation, protein and nucleic acid modification, which may be tightly linked to the initiation of RNA synthesis.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will be first described briefly.

Drawing:

Figure 1 demonstrates the ability of BMV RdRp to accurately initiate RNA synthesis from RNA or DNA proscripts. (Top) Proscript -20/13, complementary to the viral (+)-strand RNA3 from positions 1222 to 1252, contains the WT BMV subgenomic promoter directing the synthesis of a 13-nt product and serves as the WT control. The initiation nucleotide is denoted by an arrow with the sequence of the RdRp product shown above. Schematics of various constructs tested are listed and to the right are the lane numbers showing the amount of RNA synthesis relative to that from the WT control in the corresponding autoradiograph. RNA sequences are denoted by bold capital letters while DNA sequences are in lower-case letters. Nucleotide substitutions in both RNA and DNA constructs are shown below each sequence. The Δ -1g proscripts lack the 3′ terminal guanylate at position -1 relative to the initiation site. (Bottom) Autoradiographs of BMV RdRp reaction products. The amount of RNA synthesis from 25 nM of proscript -20/13 WT is shown in lanes 1 and 10. T7 generated markers containing the expected sequence of the RdRp products were used to determine the sizes of the accurately initiated 13- and 14-nt BMV RdRp products. The 14-nt product is due to a 1-nt nontemplated addition by RdRp. Reactions using 25 nM of the all deoxyribose proscript, d(-20/13), are shown in lanes 2-9. RNA synthesis and accurate initiation from proscript d(-20/13) were verified by the treatments indicated above the gel in lanes 3-5 and lanes 6-9, respectively. The amount of RdRp product from 125 nM of RNA or DNA templates with a

penultimate initiation site is shown in lanes 11-21. The treatments shown above lanes 12-14 and 19-21 demonstrate the initiation requirements from r(-1/13) and d(-1/13), respectively. The treatments indicated above lanes 16-18 verify RNA synthesis from d(-1/13). Lane ø represents the products of a control reaction with no added template while Std lanes represent products with no additional treatments.

Figure 2 displays ribose moieties which facilitate RNA synthesis by RdRp. (Top) The sequence of the -20/13 WT proscript is shown with the initiation site marked by an arrow. The sequences of hybrid proscripts, containing both ribose and deoxyribose residues, are listed below. RNA sequences are denoted by bold capital letters while DNA sequences are in lower-case letters. Proscripts containing substitutions of the 2'-OH at position -11 relative to the initiation site were constructed to determine how this functional group interacts with the BMV RdRp. The lane number containing the RdRp product generated from each proscript in the autoradiograph below is shown to the right. (Bottom) Autoradiograph of the BMV reaction products from the hybrid proscripts. The amount of RNA synthesis from 25 nM of each proscript is shown in lanes 1-11 with the percent activity of each hybrid proscript relative to that from the -20/13 WT proscript shown below the gel. Product sizes are denoted on the side. Lane ø represents the products of a control reaction with no added template. Values listed represent the mean of at least five independent experiments.

Figure 3 displays the role of ribose 2'-OHs in stable interaction with RdRp. (Top) The sequence of the -20/15 WT proscript, directing synthesis of a 15-nt product from the initiating cytidylate (arrow) is shown. Listed below this RNA construct are the sequences of various competitors all containing a WT subgenomic promoter sequence. The -20/-1 proscript contains the WT subgenomic promoter from position -20 to -1 relative the initiation site and serves as a negative control. The concentration of competitor needed to

reduce synthesis from 25 nM of the -20/15 proscript by 50% (I50) are listed to the right. (Bottom) Determination of I50 values for RNA and DNA subgenomic promoters. The amount of 15-nt product generated from the -20/15 RNA proscript was measured and plotted as a function of the concentration of each competitor. The identities of the competitors are shown to the right of the graph. Data points represent the mean of three independent standard deviations shown.

Figure 4 Minimal DNA proscripts can inhibit viral RNA synthesis *in vitro*. (Top) The sequence of the -20/15 WT proscript, directing synthesis of a 15-nt product, is shown. The arrow denotes the initiation site. Listed below are the sequences of various DNA inhibitors with successive 5′ truncations. Each of the oligonucleotides containing a WT initiation sequence is capable of directing the BMV RdRp to synthesize 13-, 8-, or 6-nt products, respectively. The d(-1/13) Rev proscript serves as a negative control. The names and I50 values are listed to the sides. (Bottom) Determination of I50 values for the DNA inhibitors. The amount of 15-nt product generated from the -20/15 RNA proscript was measured in the presence of increasing amounts of DNA templates. The I50 value was quantitated as the concentration of inhibitor needed to reduce the 15-nt product from 25 nM of -20/15 proscript by 50%. The identities of the inhibitors are shown to the right of the graph. Data points represent the mean of three independent experiments with deviations shown.

Figure 5 displays functional moieties in the subgenomic promoter required for initiation of RNA synthesis. (A) Predicted functional groups required for interaction with the BMV RdRp. The sequence shown is proscript -20/13 containing the WT BMV subgenomic promoter directing synthesis of a 13-nt product and serves as the WT control. The subgenomic initiation site is denoted by an arrow with the sequence of the RdRp product shown above. The base moieties predicted to interact with the RdRp by

previous mutational studies (Siegel et al., 1997, Proc. Natl. Acad. Sci. 94, 11238-11243) are indicated below the four nucleotides essential for RNA synthesis. (B) Recognition of the guanylate residue at positions -17 and -11. The bands denoted by asterisks represent terminal transferase labeling of the input template. (C) Recognition of the adenylate at position -14 and the cytidylate at position -13. The structures of the particular base for each position and of the nucleosides in the case of the guanylate at positions -17 and -11, are shown with arrows indicating defined changes in the functional groups mediated by the insertion of various base analogs. Numbers in parenthesis indicate the lane in the autoradiograph containing the RdRp reaction products from proscripts containing the indicated base analog. Lane WT represents the products directed by the -20/13 proscript while lane ø represents the products of a control reaction with no added template. The reaction products were separated by denaturing PAGE and visualized by autoradiography with their sizes denoted on the side. The predominant RdRp product was 14-nucleotides due to the non-templated addition of one residue at the 3′ end of the RNA product (Siegel et al., supra). Accurate initiation was verified by described enzymatic manipulations (Siegel et al., supra) and comparison to T7 generated size markers. Values listed below the gels represent the percent activity from promoters containing each base analog compared to that from the WT promoter sequence.

Figure 6 displays the template requirements for stable interaction with RdRp. (A) Schematic of proscripts containing 5′ truncations of the template. The initiation site is denoted with an arrow. Proscripts containing the +1 c/g mutation (circled) are unable to direct RNA synthesis. The names and I50 values for each construct are listed to the sides. (B) Determination of I50 values. The amount of activity from the -20/15 proscript directing synthesis of a

15-nt product was measured in the presence of increasing amounts (up to 10-fold molar excess, 250 nM) of competitor templates. The I50 value was determined as the concentration of competitor needed to reduce the 15-nt product from 25 nM of the -20/15 proscript by 50%. The identities of the various proscript competitors are labeled to the right of the graph. Data points represent the mean of at least 3 independent experiments with the standard deviation expressed as error bars.

Figure 7 demonstrates the recognition of the subgenomic initiation site. The sequence of the WT -20/13 proscript is shown with the initiation site denoted. The structure of the base analogs incorporated at positions -1, +1, and +2 are listed below with the names of the various proscripts shown on the left. The lanes containing the RdRp reaction products from the proscripts containing the base analogs are indicated to the right. Lane ø represents the products of a control reaction with no added template. The reaction products were separated by denaturing PAGE and visualized by autoradiography with their sizes denoted on the side. Values listed below the gels represent the percent activity from promoters containing each base analog compared to that from the WT promoter sequence.

Figure 8 displays a model for the interaction between the BMV RdRp and the subgenomic promoter elements needed to initiate RNA synthesis. Essential nucleotides are boxed with the key features putatively required for hydrogen bond formation with amino acid residues in RdRp shown above. The 2'-hydroxyl at position -11 also contributes to RNA synthesis. Recognition of the initiating nucleotide may occur by the rGTP primer bound by the RdRp. The oval represents a low resolution structure of the RdRp complex.

Figure 9 demonstrates a schematic representation of a nucleic acid molecule inhibiting viral replication.

Figure 10. I50 values of selected oligonucleotides.

Figure 11. Graph of I50 values of DNA inhibitors. The reaction mixture contained 25 nM of the -20/15 WT template RNA with 10 µl BMV RdRp in a 40 µl volume containing 20 mM sodium glutamate (pH 8.2), 4 mM MgCl2, 12.5 mM DTT, 0.5% (vol/vol) Triton X-100, 2 mM MnCl2, 200 µM ATP and UTP, 500 µM GTP, and 250 nM [α-32P] CTP. RdRp products from reactions containing DNA inhibitors (present at 10, 50, 100, 200 and 500-fold molar excess) were quantified and their relative activities were plotted against the amount of inhibitor present. I50 values were determined by calculating the amount of inhibitor need to reduce synthesis by 50% of the control reaction.

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs (e.g., antisense oligonucleotides, hammerhead or the hairpin ribozymes) are used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of RNA structure. The molecules of the instant invention were chemically synthesized. Oligodeoxyribonucleotides were synthesized using standard protocols as described in Caruthers et al., 1992, Methods in Enzymology 211,3-19, and is incorporated by reference.

The method of synthesis used for normal RNA including certain enzymatic nucleic acid molecules follows the procedure as described in Usman et al., 1987 J. Am. Chem. Soc., 109, 7845; Scaringe et al., 1990 Nucleic Acids Res., 18, 5433; and Wincott et al., 1995 Nucleic Acids Res. 23, 2677-2684 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale synthesis were conducted on a 394 Applied Biosystems, Inc. synthesizer using a modified 2.5

μmol scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-O-methylated nucleotides. Table 7 outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163 μL of 0.1 M = 16.3 μmol) of phosphoramidite and a 24-fold excess of S-ethyl tetrazole (238 μL of 0.25 M = 59.5 μmol) relative to polymer-bound 5'-hydroxyl was used in each coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, were 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer; detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I2, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile was used directly from the reagent bottle. S-Ethyl tetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical, Inc.

Deprotection of the RNA was performed as follows. The polymer-bound oligoribonucleotide, trityl-off, was transferred from the synthesis column to a 4mL glass screw top vial and suspended in a solution of methylamine (MA) at 65 °C for 10 min. After cooling to -20 °C, the supernatant was removed from the polymer support. The support was washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant was then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, were dried to a white powder.

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA•HF/NMP solution (250 μL of a solution of 1.5mL N-methylpyrrolidinone, 750 μL TEA and 1.0 mL TEA•3HF to provide a 1.4M HF concentration) and heated to 65°C for 1.5 h. The resulting, fully deprotected, oligomer

was quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.

For anion exchange desalting of the deprotected oligomer, the TEAB solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that was pre-washed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA was eluted with 2 M TEAB (10 mL) and dried down to a white powder.

RNA may be modified to enhance stability with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 TIBS 17, 34; Usman et al., 1994 Nucleic Acids Symp. Ser. 31, 163; Burgin et al., 1996 Biochemistry 6, 14090).

RNA was purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Stinchcomb et al., International PCT Publication No. WO 95/23225, the totality of which is hereby incorporated herein by reference) and are resuspended in water.

The sequences of the RNA that are chemically synthesized, useful in this study, are shown in figures 1-5. Those in the art will recognize that these sequences are representative only of many more such sequences where the chemical composition of the nucleotides may be altered. For example, a "cap" structure may be added to the 5′ or 3′ end to increase stability.

Administration of Nucleic Acid Molecules

Methods for the delivery of nucleic acid molecules is described in Akhtar et al., 1992, Trends Cell Bio., 2, 139; and Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995 which are both incorporated herein by reference. Sullivan et al., PCT WO 94/02595, further describes the general methods for delivery of enzymatic RNA molecules . These protocols may be utilized

for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, nucleic acid molecules may be directly delivered ex vivo to cells or tissues with or without the aforementioned vehicles. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan et al., supra and Draper et al., PCT WO93/23569 which have been incorporated by reference herein.

The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a patient.

The negatively charged polynucleotides of the invention can be administered (e.g., RNA, DNA or protein) and introduced into a patient by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions; suspensions for injectable administration; and the like.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic administration, into a cell or patient, preferably a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation to reach a target cell (i.e., a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant in vivo systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, e.g., nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of

the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as the cancer cells.

The invention also features the use of the a composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer an method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic et al. Chem. Rev. 1995, 95, 2601-2627; Ishiwata et al., Chem. Pharm. Bull. 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic et al., Science 1995, 267, 1275-1276; Oku et al.,1995, Biochim. Biophys. Acta, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu et al., J. Biol. Chem. 1995, 42, 24864-24870; Choi et al., International PCT Publication No. WO 96/10391; Ansell et al., International PCT Publication No. WO 96/10390; Holland et al., International PCT Publication No. WO 96/10392; all of these are incorporated by reference herein). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen. All of these references are incorporated by reference herein.

The present invention also includes compositions prepared for storage or administration which include a pharmaceutically effective amount of the desired compounds

in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. Id. at 1449. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used. Id.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

Vector Based Delivery of Nucleic Acid Molecules

In another aspect of the invention, nucleic acid molecules that cleave target molecules are expressed from transcription units (for example of ribozymes, see Couture et al., 1996, TIG., 12, 510) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Nucleic acid molecule expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient

expression of nucleic acid molecules. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecules bind to target viral polymerases. Delivery of nucleic acid molecule expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture et al., 1996, TIG., 12, 510).

In one aspect the invention features, an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the instant invention is disclosed. The nucleic acid sequence encoding the nucleic acid molecule of the instant invention is operable linked in a manner, which allows expression of that nucleic acid molecule.

In another aspect the invention features, the expression vector comprises: a transcription initiation region (e.g., eukaryotic pol I, II or III initiation region); b) a transcription termination region (e.g., eukaryotic pol I, II or III termination region); c) a gene encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the gene encoding the nucleic acid molecule of the invention; and/or an intron (intervening sequences).

Transcription of the nucleic acid molecules sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of

a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 Proc. Natl. Acad. Sci. U S A, 87, 6743-7; Gao and Huang 1993 Nucleic Acids Res., 21, 2867-72; Lieber et al., 1993 Methods Enzymol., 217, 47-66; Zhou et al., 1990 Mol. Cell. Biol., 10, 4529-37). This strategy may be preferable since the nucleic acid molecules of the invention would only be expressed in those cells which express the polymerase and therefore may reduce toxicity.

Transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are also useful in generating high concentrations of desired RNA molecules such as ribozymes in cells (Thompson et al., supra; Couture and Stinchcomb, 1996, supra; Noonberg et al., 1994, Nucleic Acid Res., 22, 2830; Noonberg et al., US Patent No. 5,624,803; Good et al., 1997, Gene Ther. 4, 45; Beigelman et al., International PCT Publication No. WO 96/18736; all of these publications are incorporated by reference herein. The above RNA transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, supra).

In yet another aspect the invention features an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecule of the invention, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a gene encoding at least one said

nucleic acid molecule; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another preferred embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) a gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

Examples

Brome Mosaic Virus

Brome mosaic virus (BMV) is a useful model to define the steps in RNA synthesis for (+)-strand RNA virus. BMV has three genomic RNAs, designated RNA1, 2, and 3 and a subgenomic RNA4. These RNAs encode four proteins: the

helicase-like 1a (109 kDA), the polymerase-like 2a (96 kDa), the movement protein 3a (34 kDa), and the capsid protein (20 kDa). Each BMV RNA contains a highly conserved 3 ' region which folds into a tRNA-like structure that is required to direct the synthesis of (-)-strand RNA. The (-)-strand RNA serves as template and provides cis-acting sequences for genomic (+)-strand and subgenomic RNA synthesis

The BMV RNA replication enzyme is a complex localized in the endoplasmic reticulum. It contains the BMV-encoded 1 a and 2a proteins and yet unidentified host proteins. Membrane-associated replicase can be solubilized with nonionic detergents and still retain the ability to direct synthesis of (-)-strand RNAs or subgenomic (+)-strand RNA from exogenously added genomic RNAs or (-) strand BMV RNA3, respectively. Detergent-solubilized BMV replicase, named RNA-dependent RNA polymerase (RdRp), can utilize (+)-strand RNAs of less than 160 nucleotides containing the conserved tRNA-like sequence to direct BMV specific RNA synthesis in vitro.

BMV is a positive strand RNA virus and is the type member of the bromovirus group of plant viruses in the alphavirus-like superfamily of positivesense RNA viruses. Monocistronic RNA1 and RNA2 encode proteins 1a (containing putative methyltransferase and helicase domains) and 2a (containing polymerase-like domains), respectively. In conjunction with cellular proteins, e.g., eIF3, 1a and 2a compose the template-specific BMV RdRp. The dicistronic RNA3 encodes the 3a movement protein and the coat protein, whose translation is directed by the subgenomic RNA4 (0.88 kb). Synthesis of subgenomic RNA4 is by internal initiation from a (-)-strand copy of RNA3.

The BMV RdRp complex is integrated into plant membranes but can be solubilized with high-concentration-salt and nonionic detergents, such as Triton X-100. The BMV RdRp has been highly enriched and can specifically synthesize minus-strand RNA from input plus-strand RNA templates in a

sequence-specific manner. In addition, the BMV RdRp can also synthesize subgenomic plus-strand RNA 4 by initiating with a guanylate residue internally within the minus-strand of RNA 3. The synthesis of minus-strand RNA initiates from the conserved 3' ends of plus-strand BMV RNAs. This conserved region can fold into a tRNA-like structure. As in tRNAs, the terminal three residues are 5'-CCA-3 '. Initiation of minus synthesis was reported to begin at the penultimate cytosine residue, making the first nucleotide of the newly synthesized RNA a guanylate

BMV RNA synthesis is amenable to biochemical studies because the viral RdRp can use exogenously added templates containing BMV promoter sequences. Accurate initiation of (-)-strand RNA synthesis from input (+)-strand templates has been demonstrated. Several steps in (-)-strand RNA synthesis have been defined, including initiation, primer-induced RNA synthesis, the synthesis of abortive initiation products of up to 8 nts accumulating at a 10-fold molar excess to full-length RNA (Sun *et al.*, 1996) and the transition of the RdRp from initiation to elongation (Sun & Kao, 1997a, 1997b). In contrast, the mechanism of subgenomic RNA synthesis has not been carefully studied. Short regions of (-)-strand RNA3 have been employed to refine previous characterizations of the subgenomic promoter and determine how the RdRp recognizes the promoter (Adkins *et al.*, 1997; Siegel *et al.*, 1997). As described hereinbelow, the mechanism of subgenomic (+)-strand RNA synthesis, including initiation and termination, is discerned and compared to (-)-strand synthesis.

Conservation of Polymerase Activity

As the structures of several different polymerases appear conserved, it has been suggested that the mechanism of nucleic acid synthesis is also conserved. The best characterized polymerases are the DNA-dependent RNA polymerases (DdRp) that are responsible for transcription. DNA-dependent RNA synthesis has been divided into a number

of biochemically distinct steps: binding of the DdRp to the promoter, formation of a transcriptionally active open complex, synthesis of the first phosphodiester bond, abortive RNA synthesis, promoter clearance, processive elongation and termination. The progression of these steps is accompanied by increases in the affinity of the interaction between the polymerase and the template, with commitment of the polymerase to the template taking place during/soon after the first translocation step. The committed polymerase is thought to remain stably associated with the template even though additional nucleotides needed for elongation may be lacking in the reaction).

Viral RNA replication is mediated by RNA-dependent RNA polymerases (RdRp). For a positive-sense RNA virus, the genomic (+)-strand RNA serves as a template for synthesis of (-)-strand RNA which, in turn, serves as a template for synthesis of additional copies of genomic (+)-strand RNA and, in many viruses, (+)-strand subgenomic RNAs.

Elucidating the details of RNA synthesis by RdRp may provide the foundation for studies of RNA repair and recombination and allow a comparison to RNA synthesis by DdRp. Results from previous characterization of *in vitro* RNA synthesis by the BMV RdRp defined several steps, including: (1) initiation of RNA synthesis at the penultimate cytidylate at the 3' end of BMV (+)-strand templates (Miller *et al.*,1985, *Nature (London)*, 31S, 68-70; Kao & Sun, 1996, *J. Virol.*, X, 6826-6830), (2) abortive oligoribonucleotide synthesis (Sun *et al.*, 1996, *Virology*, 296, 1-12), and (3) processive RNA synthesis (Sun & Kao.1997. *Virology*, I, 63-73). Steps in RNA synthesis by RdRp appear to mirror those seen in transcription by DdRps, including the release of abortive initiation products and the progression to elongation after the synthesis of nascent RNAs of 8 to 10 nt. This is perhaps not surprising since the catalytic subunits of all polymerases share common structural and functional motifs.

Despite the overall similarities in RNA synthesis by DdRps and RdRp, several differences should be mentioned. First, RdRp usually initiates RNA synthesis from the ends of RNA templates rather than exclusively from a promoter within a DNA molecule as does DdRp (Miller *et al.*, 1986, *J. Mol. Riol.* 18 7, 537-546; Ishihama and Nagata, 1988; Kao and Sun, 1996, , *J. Virol.*, X, 6826-6830). Second, RdRp appears to dissociate from the template during the abortive initiation step (Sun and Kao, 1997), whereas the T7 RNA polymerase remains more stably bound to supercoiled DNA, although the stability of the T7 RNA polymerase-DNA interaction is highly dependent on the structure of the template (Diaz *et al.*, 1996, *Biochemistry* 35, 10837-10843). Third, stability of the DdRp ternary complex is maintained primarily by RNA-protein and DNA-protein interactions, and not by RNA-DNA interactions (Altmann *et al.*, 1994, *Proc Natl Acad Sci U S A.* 91, 3784-3788). For RdRp, it is possible that for some viruses, an intermediate of (-)-strand RNA synthesis is a double-stranded hybrid composed of the nascent and template RNAs (Baltimore, 1968; Takeda *et al.*, 1986; Bienz *et al.*, 1992; de Graaffet *al.*, 1995). If true, then the duplex may contribute to the stability of RdRp ternary complex.

The following are non-limiting examples demonstrating the utility of the nucleic acid molecules of the instant invention. Those in the art will recognize that certain experimental conditions such as temperatures, reaction times, media conditions, transfection reagents and RNA assays are not meant to be limiting and can be readily modified without significantly altering the protocols.

Example 1: Identification and Analysis of Recognition Elements for RdRp

Accurate Subgenomic Initiation: To determine the recognition elements contained within the subgenomic core promoter of BMV, a 33 nucleotide prescript (-20/13) was constructed which contains the WT promoter sequence 20

nucleotide 3 ' of the subgenomic initiation start site in (-)-strand RNA3. Chemical synthesis of the prescripts containing base analogs were performed on a ABI 394 automated DNA synthesizer (ABI, Foster city, CA) using conventional phosphoramidite elongation cycles according to Wincott et. al., 1995.Nucleic Acids Res, 8, 1421. After subsequent aqueous methylamine and triethylamine trihydrofluoride treatment to cleave the exocyclic amino and 2'-OH protecting groups, when present, the prescripts were purified and analyzed on anion-exchange HPLC.

This prescript directs the synthesis of a 13-nt product, the first 11 nt of which are BMV sequence followed by two guanylates added by T7 RNA polymerase to allow labeling of RdRp products with [α-32P]CTP. The BMV sequence within proscript -20/13 is complementary to the viral (+)-strand RNA3 from positions 1,222 to 1,252 and serves as the WT control.

Various mutations within the promoter sequence were created. Initially, transversions, in groups of three nucleotides, were synthesized to scan the entire promoter and determine which regions were required for RNA synthesis. Positions - 17 to - 9 were identified as containing essential nucleotides because mutations in this region of the promoter reduced the ability of the BMV RdRp to initiate synthesis of subgenomic RNA to about 2% of WT activity. Mutations at nucleotide positions - 5 to -3 had a lesser effect, retaining 16% of WT activity. The three nucleotide transversions covering all other positions of the core promoter each only reduced synthesis by 50%. The predominant RdRp product from all templates was 14 nt due to the nontemplated addition of one nucleotide, a phenomenon common to all DNA-dependent RNA polymerases and the poliovirus RdRp.

Unexpectedly, subgenomic synthesis was relatively unaffected by replacement of nucleotides from -2 to the initiation site, +1. Previous work has demonstrated that

the identity of the initiating cytidylate for the subgenomic RNA4 must be maintained. The three nucleotide transversion in the -2+1 proscript places a cytidylate at position -1 and it is conceivable that the BMV RdRp is able to utilize this nucleotide for initiation of RNA synthesis directing a product of the same size as the WT proscript by either facilitating the use of a nontemplated guanylate (instead of the templated cytidylate at the +1 position) or bypassing the +I position completely. To test whether the initiation position can be moved, two additional proscripts were made. Proscript +1C/G specifically replaces the initiating cytidylate with a guanylate while leaving all other positions as WT sequence. Proscript - 1/+1 G inserts a guanylate into the initiation site and moves the WT +1 cytidylate into the +2 position. This proscript encodes a 14 nt product (15 nt with nontemplated addition) if the inserted guanylate at the +1 position is recognized by the BMV RdRp. However, if the initiation site can be shifted to the cytidylate now occupying the +2 position, then a 13-nt product (14 nt with nontemplated addition) should be observed. Accurate initiation of the RdRp products can be verified by withholding GTP which is required only for initiation or by treatment with RNase T1 which specifically cleaves after guanylates. Thus, a correctly initiated RdRp product will decrease by 1 nt after RNase T1 digestion.

The RdRp products from proscripts -2+1, - 1/+1 G. and WT -20/13 were all initiated with GTP as judged by the reactions lacking GTP and by RNase T1 treatment and are the same size (13 and 14 nt) as T7 size markers. However, no product was synthesized from the +1 G/G proscript. Thus, a cytidylate residue is required as the initiating nucleotide, confirming with earlier observations. It appears that the BMV RdRp can recognize a cytidylate at either one nucleotide 3' or 5' proximal to the original initiation site. All of the reactions demonstrate accurate initiation and validate the use of this system for characterization of the RdRp

subgenomic core promoter interaction. These experiments also confirm that the addition of the nontemplated nucleotide generating the 14-nt product occurs at the 3 ' end of the (+)-strand product.

Nucleotides Critical for Initiation of Subgenomic RNA Synthesis: Data from the three nucleotide transversion indicated that positions - 17 to - 9 contain nucleotides important for RNA synthesis. To identify the critical residues, proscripts that contain single nucleotide transversions at each position within this region and at positions - 5 to - 3 of the subgenomic core promoter were constructed. Positions - 17, - 14, - 13, - 11, - 10, and -5 were important for synthesis because transversions significantly decreased RNA synthesis by the BMV RdRp. The most critical positions, - 17, - 14, - 13, and - 11, all had activities below 3% of the WT proscript. Changes at positions - 10 and -5 were less severe, retaining 31% to 18% of WT synthesis. As a negative control, no synthesis was observed from a proscript, +1 C/G, which contains a transversion at position +1.

Proscripts containing all possible nucleotide replacements at the most critical positions, - 17, - 14, - 13, and - 11, were assayed to test the sequence specificity of these four positions. A change of the WT guanylate to uridylate (- 17 G/U) at position - 17 supported RNA synthesis at 13% of WT activity. Other nucleotide substitutions at - 17 (- 17 G/C and - 17 G/A) were unable to direct RNA synthesis. Nucleotides other than the WT sequence at positions - 14 or - 13 did not retain appreciable activity, demonstrating the importance of the adenylate at - 14 and the cytidylate at - 13. A change at position - 11 of the WT guanylate to either uridylate or adenylate (- 11 G/U or - 11 G/A) retained about 8% activity, whereas the original - 11 G/C proscript had only 1 % of the activity of the WT proscript. To further demonstrate the specific requirements of the above four positions, the WT guanylate at position - 10 was replaced

with either a uridylate or adenylate without detrimental effects. In fact, both of these mutants, - 10 G/U and - 10 G/A, resulted in slightly better promoters than the WT proscript, generating 140% of the product of -20/ 13. These results demonstrate nucleotide specificity at positions - 17, - 14, 5 - 13, and 11 of the BMV subgenomic core promoter

Competition of Wild Type Proscripts Using Single Nucleotide Base Alterations At the Contact Sites: To test this idea of nucleotide specificity, competition experiments were performed to determine if proscripts -20/13, - 17 G/C, and - 17 G/U would affect synthesis from a second proscript with the WT promoter sequence directing synthesis of a 15 nt product (designated -20/15). The concentration of the competitor templates was varied from 0.1- to 10-fold molar excess of the -20/15 proscript. Competition was visualized by a decreasing amount of the 15-nt product with a concurrent increase of the 13-nt product. As expected, proscript -20/13 competed efficiently for synthesis because it also contains a WT subgenomic promoter. Synthesis from - 20/15 was reduced 50% when -20/13 was present in an equimolar amount. In contrast, proscript - 17 G/C was not an effective competitor, decreasing synthesis from -20/ 15 by only 15% when present at an equimolar ratio. The - 17 G/U proscript inhibited synthesis from -20/15 at a reduced level (35%) from that observed with -20/13. This level of inhibition is consistent with the result that synthesis from this mutant template was diminished but not abolished. The result from this experiment is consistent with the idea that position - 17, and most likely the other key nucleotides identified by mutagenesis, is contacted by the BMV RdRp.

Alphaviral Subgenomic Promoters: A comparison of the BMV subgenomic promoter with those of other members of the alphavirus-like super-family, infecting both plants and animals, reveals a striking conservation of the four nucleotides critical for synthesis by the BMV RdRp. In addition, all of these promoters contain a pyrimidine as the

initiating nucleotide (uridylate in animal viruses and either a cytidylate or uridylate in plant viruses) with a highly conserved adenylate at the +2 position. This similarity implies that RdRps from members of the alphavirus-like superfamily recognize subgenomic promoters by a conserved mechanism (see for example Tables 1-3).

To examine the possibility that the mode of template recognition is conserved, a proscript containing the subgenomic promoter from SFV was constructed and tested its ability to be recognized by the BMV RdRp. So that the RNA product can be visually distinguished from the 13-nt product generated from the BMV proscript, the SFV proscript was designed to direct synthesis of an 11-nt product, the first 9 nt of which are WTSFV sequence followed by two guanylates added by T7 RNA polymerase to allow labeling of RdRp products with [α-32P] CTP. The SFV proscript directs the BMV RdRp to synthesize a product which is dependent upon ATP, used only as the initiating nucleotide for subgenomic synthesis from this template. The amount of synthesis was only 0.25% of the synthesis from an equimolar amount of WT -20/13 but significantly above background. This result demonstrates a heterologous interaction between an RdRp from a plant-infecting virus with an RNA template containing the subgenomic promoter from an animal-infecting virus.

Example 2: Sequence Requirements for Subgenomic RNA Synthesis

RdRp Activity Assays: BMV RdRp was prepared from infected barley essentially as described by Sun et al. (1996). RdRp preparations used in abortive initiation studies were passed through an additional PD10 (Pharmacia) gel filtration column to remove NTPs and other low molecular weight contaminants. Standard RdRp activity assays consisted of 43 I1L reactions containing 20 mM sodium glutamate (pH 8.2), 4 mM MgCI2, 12 mM dithiothreitol, 0.5% (v/v) Triton X-100, 2 mM MnCl2, 200 μM ATP, 500 μM GTP, 200

, μM UTP, 242 nM [a-32P]CTP (400 Ci/mmol, 10 mCi/mL, Amersham), equal moles (generally 1.0 pmol) template RNA, and 5- 10 μL RdRp. Reactions were incubated 90 minutes at 30°C unless indicated otherwise. Reaction products were extracted with phenol/chloroform (1 : 1, v/v) and precipitated with three volumes of ethanol and 10 μg glycogen following standard protocols (Sambrook et al., 1989).

Analysis of RdRp Products: Products from RdRp reactions were suspended in 1X denaturing loading buffer (45% (v/v) deionized formamide, 1.5% (v/v) glycerol, 0.04% (w/v) bromophenol blue and 0.04% (w/v) xylene cyanol) and denatured by heating at 90°C for 3 minutes prior to analysis by denaturing polyacrylamide gel electrophoresis. Products were analyzed on 20% or 24% acrylamide (19: 1 acrylamide: bisacrylarnide)-7M urea gels (14 x 14 x 0.05 cm) according to published procedures (Sambrook et al., 1989). In some cases, a 5% acrylamide stacking gel (2 x 14 x 0.05 cm) was used. Products from reactions containing templates directing synthesis of 198 nt or longer products were digested with 2.5 units S1 nuclease (Promega) from 10 minutes at 30°C. Denaturing loading buffer was added to S1-treated products prior to analysis by denaturing polyacrylamide gel electrophoresis on 5% acrylamide gels while native loading buffer (5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 0.04% (w/v xylene cyanol) was added to S 1 -treated products prior to analysis by non-denaturing electrophoresis on 1% agarose gels. All gels were exposed to film at -80°C and the amount of label incorporated into newly synthesized RNAs was determined with a phosphorimager (Molecular Dynamics).

Synthesis of Templates for RdRp: PCR was used to synthesize cDNA copies of either (-)-strand BMV RNA3 encompassing the subgenomic promoter or (+)-strand BMV RNA3 or RNAI encompassing the (-)-strand promoter. Pairs of synthetic oligonucleotides, one of which contained a T7

promoter, were used in PCR reactions with cDNA clones of BMV RNA3 (pB3TP8) or RNA1 (pB1TP3), respectively (Janda et al., 1987). Thirty cycles of PCR were used for amplification with Taq polymerase, with each cycle consisting of 30 s each of denaturation at 94°C, annealing at 5°C below the lowest oligonucleotide Tm and elongation at 72 °C. PCR products were purified as described above (Sambrook et al., 1989) and used as templates for in vitro transcription. The T7 DdRp was used for all transcription reactions (Ampliscribe, Epicentre) (Table 4). Synthesis of the B3-198 template has been previously described (Sun & Kao, 1997a). Molecular weight markers of 8 and 13 nts were synthesized by the protocol of Milligan et al. (1987) using oligonucleotides T7(+) and T7(-)8mer or T7(-)13mer (Table 4). Transcripts of full-length (-)- and (+) strand RNA3 were synthesized from plasmids containing the cDNA of RNA3. Transcripts of (-)-strand RNA3 with a 250 nt extension at the 5' end and (+)-strand RNA3 with a 150 nt extension at the 5' end were synthesized from plasmids containing the cDNA of RNA3 positioned 250 or 150 nt downstream of a T7 promoter, respectively

Prior to RdRp assays, transcripts were purified by anion exchange chromatography on Qiagen tip-20 columns using the manufacturer's protocol. All RdRp templates contain two non-viral guanylates at the 5' end incorporated during initiation of T7 polymerase transcription on the cDNA templates. Concentration of RdRp templates was determined by toluidine blue staining following denaturing PAGE and/or by using a spectrophotometer as previously described (Adkins et al., 1997) .

Flanking Mutations of the Initiation Cytidylate: Transversion of the initiation cytidylate to a guanylate was previously shown to abolish the ability of the proscript to direct RNA synthesis (Adkins et al., 1997; Siegel et al., 1997). However, the BMV RdRp can inefficiently initiate synthesis using a uridylate as the first templated

nucleotide (Siegel et al., 1997). To address the roles of the neighboring nucleotides, proscripts containing all possible nucleotide replacements at the +2 position were assayed to determine the requirements for this position. Mutation of the +2 adenylate to a guanylate abolished the ability of the proscript to direct RNA synthesis while a change to a cytidylate or uridylate directed 37% or 66% of the wild-type level of RNA synthesis, respectively. Thus, an adenylate at the +2 position is preferred for subgenomic RNA synthesis although no predictions can be made for the base moieties required at +2. The nucleotide requirements at the +3 and +4 positions for BMV subgenomic RNA synthesis were next evaluated. Mutation of the +3 uridylate to a cytidylate or guanylate reduced the ability of the template to direct RNA synthesis to 64% or 58%, respectively, of the wild-type level. Mutation of the +4 adenylate to a cytidylate had no adverse effect on its ability to direct RNA synthesis. Mutation of the +4 adenylate to a guanylate reduced the ability of the template to direct RNA synthesis to 57% of the wild-type level. Thus, the effect of template sequence on RNA synthesis 5 appears to decrease as the distance from the initiation nucleotide increases.

The effect of nucleotide changes at the -1 position was also determined. Mutation of the - 1 guanylate to an adenylate reduced activity to 82% of the wild-type proscript whereas a change to a uridylate had no effect, suggesting that the identity of the -1 nucleotide does not affect the efficiency of subgenomic RNA synthesis. Quite interestingly, synthesis of RdRp products from alternate initiation sites in proscripts containing -1 mutations was observed, albeit at less than 5% of the amount from the authentic initiation site. The novel 15 nt RdRp product from proscript -1G/U was apparently initiated at the -1 position using a templated uridylate. The novel 16 nt RdRp product from proscript - 1 G/A was apparently initiated at the -2 cytidylate. In proscript -1G/A, the -2 and -1 nts

are now cytidylate-adenylate, the sequence also found at the +1 and +2 positions. These results demonstrate that there is some flexibility in recognition of the initiation site by RdRp

Relaxed Requirement for the +2 nt for (-)-Strand Synthesis: To examine whether the +2 nt in the promoter for (-)-strand synthesis had a similar preference, two templates were used (B1 -242 +2 C/A and B1 -242 + 2C/U). Each template directed synthesis of a 242 nt (-)-strand RNA1 product containing a change of the +2 cytidylate to an adenylate or uridylate. Synthesis from B 1 242 +2 C/A or B 1 -242 +2 C/U was compared with synthesis from a second template (B3-198), which directed synthesis of a 198 nt (-)-strand RNA3 product, present at the same molar concentration in the same reaction. A change of the +2 cytidylate to a uridylate reduced synthesis to 31 % (similar to that previously observed by Sun et al., 1996) while a change to an adenylate increased synthesis to 157% of wild-type B1-242 and B3-198. This result suggests that the identity of the +2 nt for (-)-strand synthesis is not as critical as it is for subgenomic synthesis.

High UTP Concentration Requirement for Subgenomic RNA Synthesis: A requirement for high GTP concentration during (-)-strand RNA synthesis was previously observed (Kao & Sun, 1996). The preference for a +2 adenylate (noted above) and the inefficient synthesis of subgenomic RNA in vitro when [a-32P]UTP is used as the radiolabel instead of the usual [a-32P]CTP suggested that the second nucleotide incorporated during subgenomic RNA synthesis (UTP) may also have special requirements. To examine this possibility, the UTP requirement for subgenomic and (-)-strand RNA synthesis was compared using up/45 (directing synthesis of a 207 nt subgenomic RNA) and B3-198. Both RNAs were used by the BMV RdRp when present individually in reactions containing 50 µM UTP. Furthermore, an equimolar mixture of the two RNAs in reactions containing 50 µM UTP yielded very similar amounts

of products (Figure 2, lane 4) to the reactions containing either RNA alone demonstrating approximately equal promoter use by the BMV RdRp. Reduction of the UTP concentration to 1.6 μM decreased synthesis from both templates but by different amounts. In three independent experiments, subgenomic RNA synthesis was reproducibly more diminished than (-)-strand synthesis by 4-10 fold. In the experiment shown, (-)-strand synthesis was reduced to 13% of the levels observed at 50 μM UTP while subgenomic synthesis was reduced to undetectable levels. These results provide additional evidence for the preference of a +2 adenylate in the template for subgenomic RNA synthesis and suggest that the synthesis of the phosphodiester bond in subgenomic RNA requires high concentrations of both GTP and UTP.

Differential Primer Use During Subgenomic and (-)-Strand Genomic RNA Synthesis: Differences in the roles of the +2 nt for subgenomic and (-)-strand synthesis prompted the comparison of the use of the initiation nucleotide for these 5 two types of RNA synthesis. Since GTP is used to initiate both subgenomic and (-)-strand RNA synthesis, the effect of GTP concentration on both types of synthesis was analyzed. No difference was observed in subgenomic and (-)-strand RNA synthesis with each first being detected at 25 μM and continuing to increase through 200 μM GTP. Next, the ability of mono- or dinucleotide primers to replace GTP as the initiation nucleotide was examined. Primers have been previously demonstrated to alleviate the need for high concentrations of GTP (Kao & Sun, 1996). Primer GpU is complementary to the initiation sequence for subgenomic RNA synthesis while GpG is complementary to the initiation sequence for (-)-strand RNA synthesis. GDP is expected to serve as a primer for both subgenomic and (-)-strand RNA synthesis

Control reactions contained 200 μM GTP and equimolar mixtures of templates for subgenomic (up/45) and genomic (-)-strand (B3-198) synthesis and resulted in synthesis of

approximately equal molar amounts of subgenomic and (-)-strand products. When GTP was reduced to 4 μM, synthesis of both subgenomic and (-)-strand products decreased to 1.5% of that observed at 200 μM GTP. The addition of GpG to reactions to final concentrations of 250-1250 μM stimulated (-)strand synthesis from 7 to 10-fold over the basal level while subgenomic RNA synthesis remained unchanged. The addition of GpU to reactions at the same concentrations stimulated subgenomic synthesis by about 3-fold over the basal level while (-)-strand synthesis was unchanged. The reduced stimulation of subgenomic as compared to (-)-strand RNA synthesis is consistent with previous observations (Kao & Sun, 1996). The subgenomic product reproducibly migrated to a lower position when primed with GpU, perhaps due to the lack of 5' phosphates on the dinucleotide primed product. The addition of GDP to reactions at 250-1250 μM stimulated synthesis of subgenomic RNA by 1.2 to 3.4 fold and (-)-strand by 9.4 to 19 fold. ADP was added at 250-1000 μM and resulted in a 1.7-fold stimulation of (-)-strand synthesis at the 1000 I1M level while no detectable increase in subgenomic synthesis was observed at any level. These results demonstrate that mono- and dinucleotide primers stimulate (-)-strand genomic RNA synthesis more than subgenomic RNA synthesis under conditions of limiting GTP. Furthermore, these results confirm the observation that the initiation of subgenomic and (-)-strand RNA synthesis has different requirements

Abortive Initiation During Subgenomic RNA Synthesis: Synthesis of abortive products during initiation of (-)-strand synthesis was previously observed (Sun et al., 1996; Sun & Kao, 1997b). Since (-)-strand synthesis initiates near the 3 ' end of the genomic RNA, it is of interest to determine whether abortive initiation occurs during initiation from an internal promoter. Thus, the products of subgenomic RNA synthesis reactions were analyzed for the presence of oligonucleotides (potentially representing

abortive initiation products) using high resolution polyacrylamide gels. [α-32P]ATP was used as a label in these experiments due to the lack of cytidylates in the expected product prior to position +14 and the inefficient labeling observed with UTP (noted above). Greater synthesis of full-length products was consistently observed with [α-32P]CTP than with [α-32P]ATP, perhaps due to ATP hydrolysis by the BMV 1 a helicase-like protein, a component of RdRp. Several sizes of oligonucleotides were observed during synthesis from proscript 12/26 (containing an 8 nt polyuridylate tract and directing synthesis of a 26 nt subgenomic product) and also during synthesis of full-length subgenomic RNA from (-)-strand RNA3. The oligonucleotides were 6, 7, and 9 nts in size by comparison to the T7 DdRp-generated RNAs of the sequences 5' GUAUUA 3', 5' GUAUUAA 3' and 5' GUAUUAAUA 3'. The RdRp-produced oligonucleotides of 6, 7 and 9 nt were in 12, 7 and 3-fold molar excess, respectively, to the full-length 26 nt product and in 20, 8 and 7-fold molar excess, respectively, to the full-length subgenomic RNA as determined by phosphorimager quantitation. Other sizes of oligonucleotides were present but observed less reproducibly.

While some endogenous BMV RNA was present in the RdRp preparation and directed synthesis of high molecular weight products in the absence of added template, no oligonucleotides were synthesized unless a (-)-strand RNA3 template was added. The oligonucleotide products were judged to be correctly initiated based on the following lines of evidence. Labeling of both oligonucleotides and full-length products was significantly reduced or eliminated when GTP, the initiating nucleotide, was omitted from the reactions. Correctly initiated RNAs do not contain a cytidylate until position +14. Thus, abortive products should lack cytidylates. It was found that oligonucleotide synthesis was unaffected when CTP was omitted from reactions while synthesis of full-length products was abolished

(although some higher molecular weight products, 14 and 17 nts, were seen presumably due to contaminating CTP). Full-length products and some potential pause products were labeled using [α-32P]CTP while the oligonucleotides were not. Finally, a proscript (37/26) containing a transversion of the +1 cytidylate to guanylate failed to direct synthesis of either oligonucleotide or full-length products.

Both the full-length 26mer and a prematurely terminated 24mer were observed from the 12t26 template when [α-32P]ATP was used as label. This is likely due to limiting ATP (242 nM) in the form of [α-32P]ATP. This observation raised the concern that limiting ATP might be responsible for the production of the oligonucleotides. To examine this possibility, successively higher concentrations of unlabeled ATP were added to RdRp reactions. Synthesis of the oligonucleotides and elongated products responded in a similar manner to the addition of increasing amounts of unlabeled ATP. The 9 nt RNA and elongated products were detectable when the ATP concentration was increased to 30 μM indicating that the synthesis of oligonucleotides is an innate property of the BMV RdRp and not due simply to limiting substrates.

Magnesium is required for the elongation phase of (-)-strand RNA synthesis while manganese suffices for initiation (Sun et al., 1996). The effect of manganese on the synthesis of oligonucleotide and full-length products from the subgenomic promoter was examined. At 1 to 2 mM, MnCl2 increased synthesis of full-length products by 8-28% and the synthesis of the 9 nt RNA by 95- 127%. Addition of MnCl2 to more than 2 mM reduced synthesis of both oligonucleotides and elongated products although elongated products were more sensitive at lower MnCl2 concentrations. The addition of MnCl2 also resulted in the appearance of a novel oligonucleotide product. These results correspond to previous observations for initiation of (-)-strand RNA synthesis (Sun et al., 1996).

To determine whether the oligonucleotides produced during subgenomic RNA synthesis were released by RdRp and thus represented abortive products, the following experiments were conducted. Reactions lacking CTP should arrest RdRp on the template RNA. These arrested complexes were fractionated by passage through Sephadex CL-6B spin columns. Sixteen consecutive fractions were collected and divided into two sets, one of which was analyzed for RNA products and the second of which was assayed for RdRp activity. Elongated RNAs and RdRp activity were found in fractions 2 and 3 while the 6, 7 and 9 nt oligonucleotides were found in fractions 8 to 13. These results demonstrate that the elongated RNAs remain in a ternary complex with the RdRp while the oligonucleotides are released from the RdRp complex and hence represent the products of abortive initiation.

Example 3: Inhibition of RNA Synthesis Using a Competitor Nucleic Acid Molecule

To determine whether short RNAs corresponding to the initiation sequence can inhibit RNA synthesis, an *in vitro* assay was employed (Table 4). The reaction was performed with a tester template and increasing concentrations of the competitor RNA. The results were then plotted as the percentage of activity of the tester template in the presence of increasing concentrations of inhibitors. This plot yielded thVe inhibitor concentration needed to reduce synthesis by 50% (I_{50})

A competitor RNA of 33 nts (wt-13) which contained the wild-type promoter and initiation sequence reduced the synthesis from the tester template (wt-15). The concentration for the wt-13 RNA needed to reduce wt-15 synthesis to 50% (I_{50}) was 20 nM. To determine the length required for efficient inhibition of RNA synthesis from the tester template, an RNA of 23 nt containing the promoter and an additional three nucleotides was employed. This RNA

had an I_{50} of 80 nM. The control, an RNA which did not contain an initiation nucleotide (-20/-1), had an I_{50} much greater than 250 nM. Moreover, no significant inhibition was observed with this template even when it was present at 250 nM. These results suggest that relatively low concentrations of short RNAs are potent inhibitors of RNA synthesis by the viral RdRp. An 8 nt competitor DNA molecule containing the initiation nucleotide, but lacking the promoter sequence, also competed for synthesis from the 25 tester template. This 8 nt DNA has an I_{50} of 2.5 micromolar. (Figure 10 and 11) This result also indicates that single stranded DNA can be used to inhibit viral replication, an observation that is of interest for pharmaceuticals, given the inherently increased stability of DNA molecules in human serum.

Example 4: RdRp Mediated Synthesis of RNA Using a DNA Template

RdRp's ability to recognize and accurately initiate RNA synthesis from a DNA version of the subgenomic promoter was tested. As the WT control, a 33-nt proscript (designated -20/13) was constructed which contains the WT promoter sequence directing the synthesis of a 13-nt product, the first 11-nts of which are BMV sequence followed by two guanylates which allow labeling of RdRp products with [a-32 P] CTP (12). Standard assays consisted of 25 nM of proscript RNA (unless stated otherwise) with 10 µl of RdRp in a 40 µl reaction containing 20 mM sodium glutamate (pH 8.2), 4 mM MgCl2, 12.5 mM dithiothreitol, 0.5% (v/v) Triton X-100, 2 mM MnCl2, 200 µM ATP and UTP, 500 µM GTP, and 250 nM [a-32 P]CTP (Amersham). Reactions were incubated at 30°C for 90 minutes and stopped by phenol/chloroform extraction followed by ethanol precipitation in the presence of 5 µg of glycogen and 0.4 M ammonium acetate. Products were separated by electrophoresis on 20% denaturing (8 M urea) polyacrylamide gels. Gels were wrapped in plastic and exposed to film at -

80°C. Product bands were quantified using a Phosphorimager (Molecular Dynamics).

As judged from T7 DdRp generated size markers, the predominant RdRp product was 14-nts due to the nontemplated addition of one residue, a phenomenon common to many polymerases (Fig. 1, lanes 1 & 10).

The all DNA proscript, designated d(-20/13), inserted deoxyriboses in every position while still containing otherwise WT subgenomic promoter and template sequences. This construct was able to direct RNA synthesis by RdRp (Fig. 1, lanes 2-8); however, the predominant product was now 13-nts rather than 14-nts as it was with the RNA template. This change may reflect the need for 2'-OHs in the template in order to efficiently add the nontemplated nucleotide. Product synthesis was resistant to inhibitors of DNA-dependent RNA polymerases, such as actinomycin D and rifampicin. Pretreating the d(-20/13) proscript with DNase I abolished product synthesis. The product, however, was resistant to DNase I while completely sensitive to RNase A (Fig. 1, lanes 3-5). Accurate initiation was verified in a number of ways: comparisons of the product sizes to those from the -20/13 WT proscript (Fig. 1, lane 1 vs. 2), RNase T1 digestion (which cleaves after the initiating guanylate) resulted in labeled products 1-nt smaller than those without digestion (Fig. 1, lane 6 vs. 7), the absolute requirement for GTP which is only needed for initiating accurate synthesis (Fig. 1, lane 8), and the lack of synthesis from a proscript with a mutant initiation site (Fig. 1, lane 9). Although the amount of RNA synthesis was approximately 6% relative to that from the WT RNA proscript, these results conclusively demonstrate that the BMV RdRp was able to productively interact with this DNA construct.

Recognition of the Terminal Initiation Site: The ability for RdRp to recognize a terminal initiation site from a DNA template (Figure 1) was tested. Constructs retaining nucleotides at positions -1 to +13 relative to the

initiation site were synthesized in both RNA and DNA versions, r(-1/13) and d(-1/13) respectively. Removal of the subgenomic promoter from positions -20 to -2 places the initiation site at the penultimate position, mimicking the preferred position for initiation from the 3' end of viral templates (Miller *et al.*, 1985 *Nature* 313, 68). Both versions of the -1/13 proscript were able to direct RNA synthesis by RdRp at approximately equal levels (6% vs. 8%, respectively, relative to the amount of synthesis from the -20/13 WT proscript). As observed for proscripts containing the subgenomic promoter, the predominant product was 14-nts for the RNA template, r(-1/13), and 13-nts for the DNA template, d(-1/13).

The requirements for initiation from these templates were determined by Mutations within the initiation site were generated to test the requirements for initiation from these templates. Mutation of the +1 cytidylate or removal of the -1 guanylate abolished RNA synthesis in both r(-1/13) and d(-1/13) templates (Fig. 1, lanes 12 & 14; 19 & 21, respectively), demonstrating that initiation must occur from a cytidylate at the penultimate position as it does for full-length genomic synthesis. However, RdRp differed in its ability to tolerate the change of the +2 adenylate to a cytidylate in these templates. The +2 a/c mutation abolished the ability to direct RNA synthesis in the r(-1/13) template (Fig. 1, lane 13); whereas, RNA synthesis was unaffected by this mutation in the d(-1/13) template (Fig. 1, lane 20), indicating a difference in the mode of recognition by RdRp. RNA synthesis from the d(-1/13) template was verified as above; treatment with DNase I degraded the DNA template and abolished RNA synthesis while the product was resistant to DNase I, but degraded by RNase A (Fig. 1, lanes 16-18). The RdRp from healthy tomato leaves has also been observed to initiate RNA synthesis from the end of a DNA template, but this initiation did not occur in a sequence-specific manner nor were the requirements for

initiation fully characterized (Schiebel *et al.*, 1993, *J Biol Chem* 268, 11858).

Initiation Requirements: The requirements for initiation from these templates were determined by mutations within the initiation site were generated to test the requirements for initiation from these templates. Mutation of the +1 cytidylate or removal of the -1 guanylate abolished RNA synthesis in both r(-1/13) and d(-1/13) templates (Fig. 1, lanes 12 & 14; 19 & 21, respectively), demonstrating that initiation must occur from a cytidylate at the penultimate position as it does for full-length genomic synthesis. However, RdRp differed in its ability to tolerate the change of the +2 adenylate to a cytidylate in these templates. The +2 a/c mutation abolished the ability to direct RNA synthesis in the r(-1/13) template (Fig. 1, lane 13); whereas, RNA synthesis was unaffected by this mutation in the d(-1/13) template (Fig. 1, lane 20), indicating a difference in the mode of recognition by RdRp. RNA synthesis from the d(-1/13) template was verified as above; treatment with DNase I degraded the DNA template and abolished RNA synthesis while the product was resistant to DNase I, but degraded by RNase A (Fig. 1, lanes 16-18). The RdRp from healthy tomato leaves has also been observed to initiate RNA synthesis from the end of a DNA template, but this initiation did not occur in a sequence-specific manner nor were the requirements for initiation fully characterized (Schiebel *et al.*, *supra*).

Hybrid Templates: Hybrid proscripts, containing both ribose and deoxyribose residues, were generated to determine the locations of residues that facilitate RNA synthesis by RdRp (Fig. 2). Hybrid H1, containing riboses only in the subgenomic promoter and the +1 and +2 positions, directed an increased amount of RNA synthesis (20%) relative to the d(-20/13) proscript (6%). However, synthesis was still below that obtained from the -20/13 WT proscript (Fig. 2, lanel vs. 2), indicating a preference for ribose residues in the

template portion of the proscript. Replacement of the deoxyuridines with deoxythymidines, containing a methyl group at the C5 position of the base, in the template from positions +2 to +13 in hybrid H4 did not appreciably alter RNA synthesis relative to the H1 proscript (Fig. 2, lane 5).

RNA synthesis comparable to that from H1 was observed from hybrids that contained an increasing amount of deoxyriboses within the subgenomic promoter. Hybrid H2 extended the region of deoxyribose replacement, confirming that riboses at positions +1 and +2 were not important for RNA synthesis (Fig. 2, lane 2 vs. 3). H3, which contains deoxyriboses at every position except those at -17, -14, -13, -11, +1, and +2, directed a similar amount of RNA synthesis as that from the H1 proscript (Fig. 2, lane 2 vs. 4). The results from hybrids H2 and H3 indicated that ribose residues in the subgenomic promoter may only be required at positions -17, -14, -13, -11 or a subset thereof. Previously (11), applicant had found that the placement of a deoxyguanosine at position -17 in an otherwise RNA proscript had no effect on RNA synthesis, but a deoxyguanosine at position -11 reduced synthesis by over half relative to the -20/13 WT control (Fig. 2, lanes 6 & 7, respectively). These results identified the ribose at position -11 as being important for RNA synthesis.

Sugar Chemical Modifications: RdRp could recognize the 2'-OH of the ribose at position -11 either as a hydrogen bond donor, as a H bond acceptor, or by the orientation of the ribose which is affected by the C2' moiety. To determine the role of the 2'-OH at position -11, applicant synthesized DNA proscripts of WT sequence, d(-20/13), with various C2' substitutions (14). The -11 sugar containing a 2'-OH (ribose), 2'-OCH3 and 2'-F were chosen since these substitutions should be able to form the C3' endo conformation (16). Also tested was 2'-NH2 which forms the C3' endo conformation at a reduced frequency. Potential H-bond acceptors include: 2'-OH, 2'-NH2, 2'-OCH3 and 2'-F

while the 2'OH and 2'-NH2 can also act as hydrogen donors. The amount of RNA synthesis from the proscripts containing these replacements were determined (Fig. 2, lanes 8-11). Each C2' substitution at position -11 was able to direct RNA synthesis 2- to 3-fold better than the all DNA proscript, d(-20/13), confirming the importance of the 2'-position at the -11G residue. Surprisingly, these substitutions had similar abilities to direct RNA synthesis relative to one another (ranging from 10% to 16%). The level of RNA synthesis from the proscript containing the 2'-NH2 (Fig. 2, lane 9) indicates that a predominant C3' endo orientation at the -11G residue may not critical for proper interaction with RdRp. Since the 2'-H is the only substitution which cannot accept a hydrogen bond, a possible explanation for the importance of the -11 ribose is that it provides a hydrogen bond acceptor site which is required for the proper positioning of RdRp at this position. Alternatively, the presence of the 2'-OH may prevent some unknown deleterious structure from occurring by steric interference.

Competition Assay: A template competition assay was used to evaluate whether the insertion of deoxyriboses in the subgenomic promoter had an adverse effect on the ability to be directly bound by RdRp as would be expected from the functional results (Fig. 3). The amount of synthesis from a WT promoter directing the production of a 15-nt product (proscript -20/15) was determined in the absence and presence of various competitor templates. The concentration of the competitor required to reduce the activity from the -20/15 proscript by 50% was termed the I50 value. Competitors able to interact more strongly with RdRp will better reduce synthesis from -20/15, resulting in lower I50 values.

Proscript -20/13 WT (composed entirely of ribose residues) reduced the level of 15-nt synthesis by half when present in the same molar ratio as the -20/15 proscript, generating an I50 of 25 nM (Fig. 3). The ability of d(-

20/13) to be bound by RdRp was only mildly affected, having an I50 value of 90 nM (Fig. 3). This 3- to 4-fold reduction in I50 value was surprising given that d(-20/13) was reduced in the ability to direct RNA synthesis by over 15-fold relative to that from -20/13 WT proscript. The presence of either a -OH or -OCH3 group at the C2′ position of the -11 guanylate in an otherwise all deoxyribose proscript virtually restored the ability to be bound by RdRp, as indicated by I50 values of 30 nM (Fig. 3). The fact that the same level of binding was observed with the 2′-OCH3 substitution as was seen with 2′-OH suggests that this increase may be due to the restoration of a hydrogen bond contact. As a negative control, a ribose proscript containing the WT sequences from position -20 to -1 was not able to effectively inhibit 15-nt synthesis in the range of competitor tested (10-fold molar excess).

The results obtained with the constructs in Figure 1 suggested that DNA constructs of minimal lengths could be used as potential inhibitors of viral synthesis. DNA inhibitors, containing a WT initiation sequence beginning at position -1 with increasing truncations at their 5′ ends, were tested in template competition assays. As expected, all constructs were found to direct 13-, 8-, or 6-nt RNA products. All of these constructs also effectively reduced synthesis from the RNA -20/15 proscript in a manner dependent on the length of the 5′ sequence (Fig. 4). As a negative control, the d(-1/13) Rev proscript which does not contain the WT initiation sequence was not able to inhibit synthesis over the range of inhibitor tested (500-fold molar excess). The sequence-specific reduction of viral RNA synthesis *in vitro* by relatively stable DNA inhibitors should allow the rational design of viral therapeutics.

Applicant has demonstrated that RdRp has the ability to recognize and initiate accurate RNA synthesis from either an internal or terminal initiation site on a DNA template. Moreover, the functional and binding data from chemically

synthesized proscripts suggest that the 2'-OH at position -11 may be involved in hydrogen bonding with RdRp during the initiation of RNA synthesis, either directly or through a water molecule. However, ribonucleotides in the template portion of the proscript (positions +3 to +13) may be needed to direct WT levels of RNA synthesis, perhaps by stabilizing the conformational change in the polymerase as it translocates out of the initiation stage (17). This preference for template riboses is not observed in the minimal proscripts initiating synthesis from the penultimate nucleotide perhaps due to RdRp being able to adjust its conformation and/or activity in the presence of the core promoter. Other proteins which recognize specific RNAs also tend to have limited contact requirements for riboses. Examples include the MS2 coat protein and *E. coli* alanine-tRNA synthetase (18). Since the modern RdRps (or a conserved vestige thereof) best reflect the primitive RNA replicase, these results argue that no significant decrease in binding energy would have occurred during the transition from RNA to DNA templates. The removal of this potential penalty would increase the ease by which an ancestral RdRp could evolve to transcribe and perhaps replicate DNA genomes required in the emerging DNA world.

Example 5: Effects of the insertion of Chemically Modified Bases into the Template

Synthesis of proscripts: Polymerase chain reaction (PCR) was used to generate cDNA copies of the (-)-strand BMV RNA3 encompassing the subgenomic promoter from the cDNA clone of RNA pB3TP8 (9). Pairs of primers, one of which contained a T7 promoter, allowed proscript RNAs to be generated using T7 RNA polymerase (Ampliscribe, Epicentre) as described previously (5). Transcription reactions which incorporate inosines were preformed with 2 mM of the primer GpG which allows initiation to take place and ITP in place of GTP in the reaction. RNAs were purified with Qiagen columns (Chatsworth, CA) using the manufacturer's protocol

to remove NTPs and proteins remaining from the T7 transcription reaction. RNAs were visually inspected by denaturing polyacrylamide gel electrophoresis (PAGE) and quantified by UV absorbance.

Synthesis of 2'-O-TBDMSi-3'-O-phosphoramidites of purine riboside (Liu *et al.*, 1997, *J. Mol. Biol.* 267, 163-171), 2-amino purine riboside (Konforti *et al.*, 1998, J. Mol. Cell 1,433-441), and pyrimidine-2-one riboside (Murray *et al.*, 1995, Biochem. J. 311, 487-494)) were performed as previously described. 3'-O-Phosphoramidite of 7-deaza-2'-deoxyguanosine was purchased from Glen Research (Sterling, VA). Chemical synthesis of the proscripts containing these base analogs were performed on a ABI 394 automated DNA synthesizer (ABI, Foster City, CA) using conventional phosphoramidite elongation cycles according to Wincott *et. al., supra.* After subsequent ethanolic ammonium hydroxide and triethylamine trihydrofluoride treatment to cleave the exocyclic amino and 2'-OH protecting groups, the proscripts were purified and analyzed by anion-exchange HPLC (Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684). Mass spectral analysis of each chemically synthesized proscript was performed on a Voyager-DE MALDI-TOF spectrometer (Perseptive Biosystem, Framingham, MA) and all were within 0.1% of the expected mass (Table 5).

RdRp activity assay and product analysis: BMV RdRp was prepared from infected barley as described previously (Sun *et al.*, 1996, *Virology* 226, 1-12). Standard assays consisted of 25 nM of template RNA (unless stated otherwise) with 10 µl of RdRp in a 40 µl reaction containing 20 mM sodium glutamate (pH 8.2), 4 mM MgCl2, 12.5 mM dithiothreitol, 0.5% (v/v) Triton X-100, 2 mM MnCl2, 200 µM ATP and UTP, 500 µM GTP, and 250 nM [α-32 P]CTP (Amersham). Reactions were incubated at 30°C for 90 min and stopped by phenol/chloroform extraction followed by ethanol precipitation in the presence of 5 µg of glycogen and 0.4 M ammonium acetate. Products were separated by electro-

phoresis on 20% denaturing (8 M urea) polyacrylamide gels. Gels were wrapped in plastic and exposed to film at -80°C. Product bands were quantified using a Phosphorimager (Molecular Dynamics) and values were compared to the amount of product generated from the WT template (-20/13) to derive the relative percent activity of mutant templates. All values shown represent the mean of at least three independent experiments with standard deviations shown. WT -20/13 contains sequence complementary to viral (+)-strand RNA3 from positions 1222 to 1252.

Template competition assays were performed under the reaction conditions stated above except that 25 nM of proscript -20/15, directing synthesis of a 15-nt product, was incubated with increasing concentrations of various competitors. The amount of RdRp used in these reactions is limiting as previously demonstrated (Siegel *et al.*, 1997, *Proc. Acad. Natl. Sci. USA* 94, 11238-11243). The product generated from the -20/15 proscript was quantitated as above and plotted against the concentration of competitor to determine the concentration of competitor needed to reduce the15-nt product by 50%, designated as the I50 value.

Nucleotides located at positions -17, -14, -13, and -11 relative to the subgenomic initiation site (+1) were required for RNA synthesis and predictions were made regarding the functional moieties mediating this effect (Fig. 5A) (Siegel *et al., supra*). RNAs containing base analogs at each of the four critical positions were made to determine the importance of these functional groups (Fig. 5). As the WT control, a 33-nt proscript (designated -20/13) was constructed which contains the WT promoter sequence 20-nts 3′ of the subgenomic initiation start site in (-)-strand RNA3. This proscript directs the synthesis of a 13-nt product, the first 11-nts of which are BMV sequence followed by two guanylates which allow labeling of RdRp products with [α-32 P] CTP (Fig. 5B and Fig 5C, lane WT).

Applicant first examined the recognition of the guanylate at position -17 (Fig. 5B). The C6 keto (and possibly the N1) group was predicted to interact with the BMV RdRp. A proscript RNA containing the base analog 2-aminopurine which removes the C6 keto group and forms a double bond with the N1 moiety decreased RNA synthesis to background levels (Fig. 5B, lane 1). The mutational analysis did not implicate roles for the C2 amine and the N7 imine (Siegel *et al., supra*). The more limited role of the C2 amine in interaction with RdRp can be surmised by the severity of the 2-aminopurine substitution which indicates that the C6 keto and possibly the N1 groups are the determining moieties. As negative control, a change of the -17 ribose to a deoxyribose was unaffected in RdRp recognition (Fig. 5B, lane 2). Lastly, a direct examination of he N7 imine by a replacement with a carbon along with a change of the ribose to deoxyribose had no effect on the ability of the proscript to direct RNA synthesis by RdRp (Fig. 5B, lane 3).

The guanylate at position -11 was predicted to be recognized in a bidentate fashion mediated by both the C6 keto (along with the N1 amine) and the N7 imine. Incorporation of the 2-aminopurine base analog (Konforti *et al., supra*) at this position significantly decreased the ability to direct RNA synthesis to 40% (Fig. 5B, lane 4). However, RNA synthesis was not reduced to background levels, consistent with the prediction that the N7 imine was also important. The N7 deaza base analog was only available in a deoxyribose form; therefore, a control RNA with deoxyguanosine at position -11 was first tested. A deoxyribose at position -11 reduced RNA synthesis to 46% of wildtype (Fig. 5B, lane 5), implicating that the RNA backbone does mediate some aspect of subgenomic promoter recognition. Removal of both the N7 imine and the 2'-OH further reduced synthesis to 25% of wildtype, indicating recognition of the predicted N7 imine (Fig 5B, lane 6).

Again RNA synthesis was not abolished since this base analog retained the C6 keto group.

A likely candidate for RdRp recognition of the adenylate at position -14 is the exocyclic C6 amine group. To test the importance of this group, a purine riboside analog (Liu *et al., supra*) was substituted for the adenylate at this position. An RNA with this change retained synthesis at 60% of wildtype, suggesting some other features of the adenylate are important for recognition by RdRp (Fig 5C, lane 1). In contrast, the -13 cytidylate was predicted to interact with RdRp by the exocyclic C4 amine group. A proscript containing the base analog pyrimidine-2-one (12) which specifically removes this functional group reduced the level of RNA synthesis to 7% (Fig. 5C, lane 2).

RdRp contacts within the subgenomic promoter: A template competition assay was used to test whether various promoter mutations affected the ability of the RNA to interact with the BMV RdRp (Table 6). The amount of synthesis from a WT promoter directing the production of a 15-nt product (proscript -20/15) was determined in the absence and presence of various competitor templates. If a mutant proscript used as a competitor had lost its ability to be recognized by RdRp, then its presence in a reaction should not adversely affect the amount of synthesis from the WT -20/15 proscript.

As a positive control, the WT proscript -20/13 (generating a 13-nt product) was tested for its ability to inhibit synthesis of the 15-nt product from -20/15. When this proscript was present in the same molar amount as proscript -20/15, the level of 15-nt synthesis was reduced by half. Mutations at positions -14, -13, and -11 which abolished the ability to direct synthesis did not inhibit the BMV RdRp from productively interacting with a WT promoter even when present in molar excess (Table 6); the same result as was obtained with mutations at position -17 (Siegal et al., supra). In contrast, a change of the

24. The expression vector of claim 21, wherein said expression vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an intron;

d) a gene encoding at least one said nucleic acid molecule; and

wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

25. The expression vector of claim 21, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an intron;

d) an open reading frame;

e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

26. A cell comprising the nucleic acid molecules of claim 1.

27. The cell of claim 26, wherein said cell is a plant cell.

28. The cell of claim 26, wherein said cell is an animal cell.

29. The cell of claim 26, wherein said cell is a bacterial cell.

30. The cell of claim 26, wherein said cell is a mammlian cell.

31. The mammalian cell of claim 30, wherein said mammalian cell is a human cell.

32. A pharmaceutical composition comprising the nucleic acid molecule of claim 1.

33. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule is chemically synthesized.

34. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule is enzymatically synthesized.

35. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule is in a purified form.

36. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule comprises a viral nucleic acid sequence which includes the initiation nucleotide.

37. The nucleic acid molecule of claim 36, , wherein said nucleic acid molecule comprises at least four nucleotides.

38. The nucleic acid molecule of claim 1, wherein said viral polymerase is encoded by a (+) single stranded RNA virus.

39. The nucleic acid molecule of claim 1, wherein said viral polymerase is encoded by a (-) single stranded RNA virus.

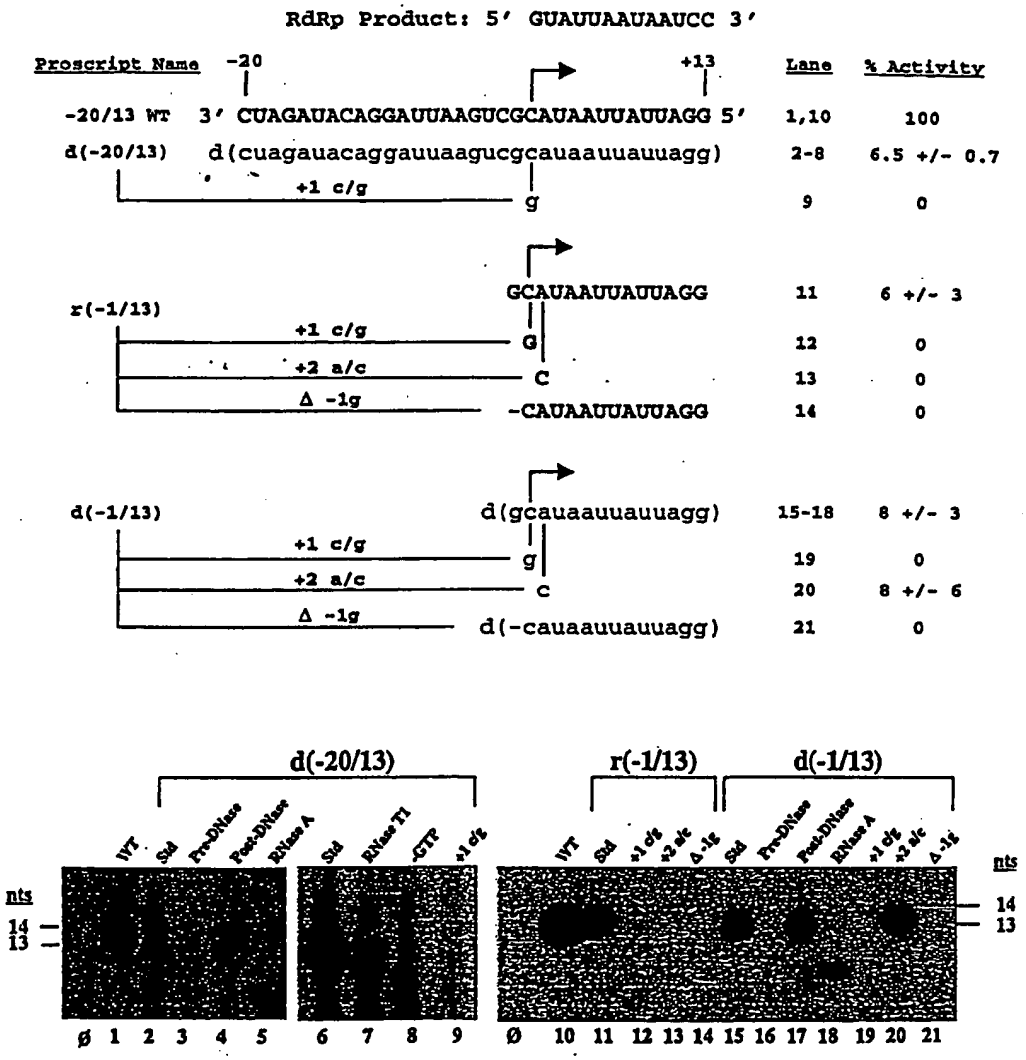


Figure 1.BMV RdRp Accurately Initiates RNA Synthesis From RNA or DNA Proscripts.

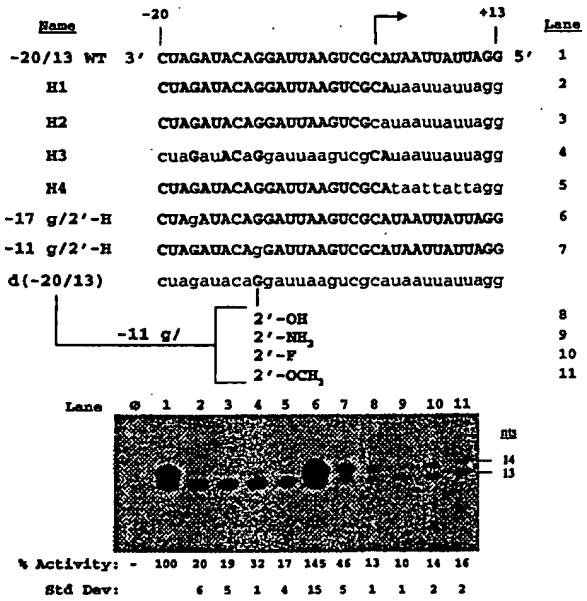
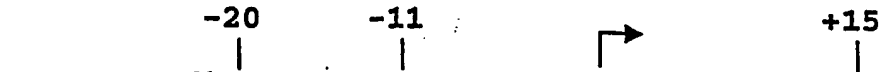
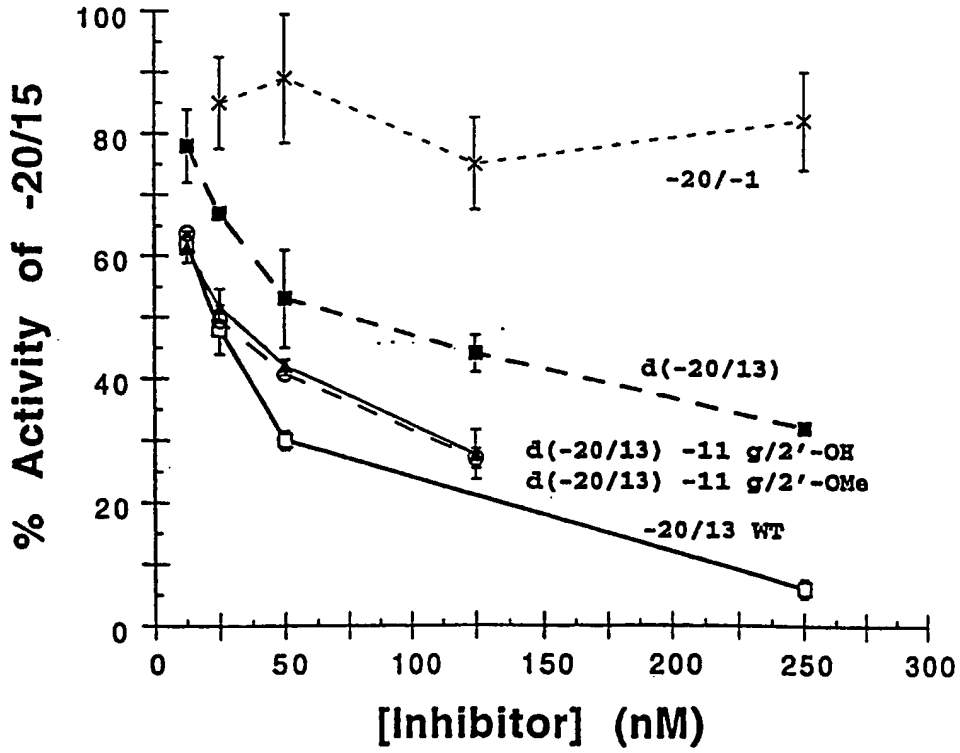


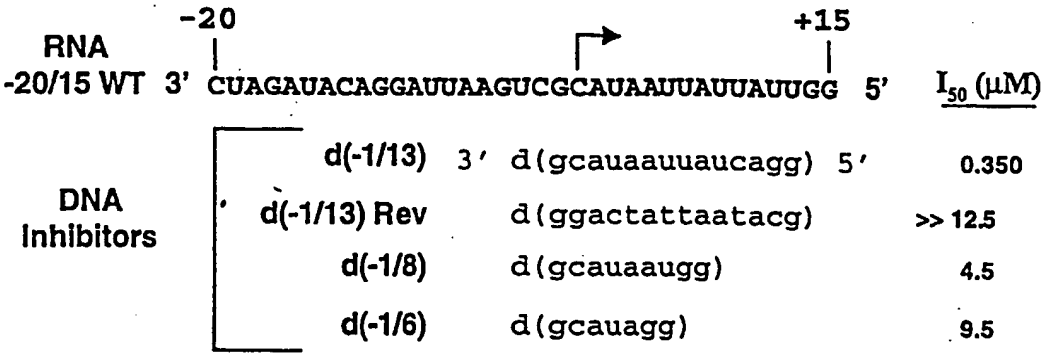
Figure 2. Ribose moieties which facilitate RNA synthesis by RdRP

Figure 3.Role of Ribose 2′-OHs in Stable Interaction with RdRp.



Competitors		I_{50} (nM)
-20/13 WT	CUAGAUACAGGAUUAAGUCGCAUAAUUAUUAGG	25
d(-20/13)	d(cuagauacaggauuaagucgcauaauuauuagg)	75
d(-20/13)	d(cuagauacaGgauuaagucgcauaauuauuagg)	
	-11 g/ 2′-OH	25
	-11 g/ 2′-OCH_3	25
-20/-1	CUAGAUACAGGAUUAAGUCG	>>250





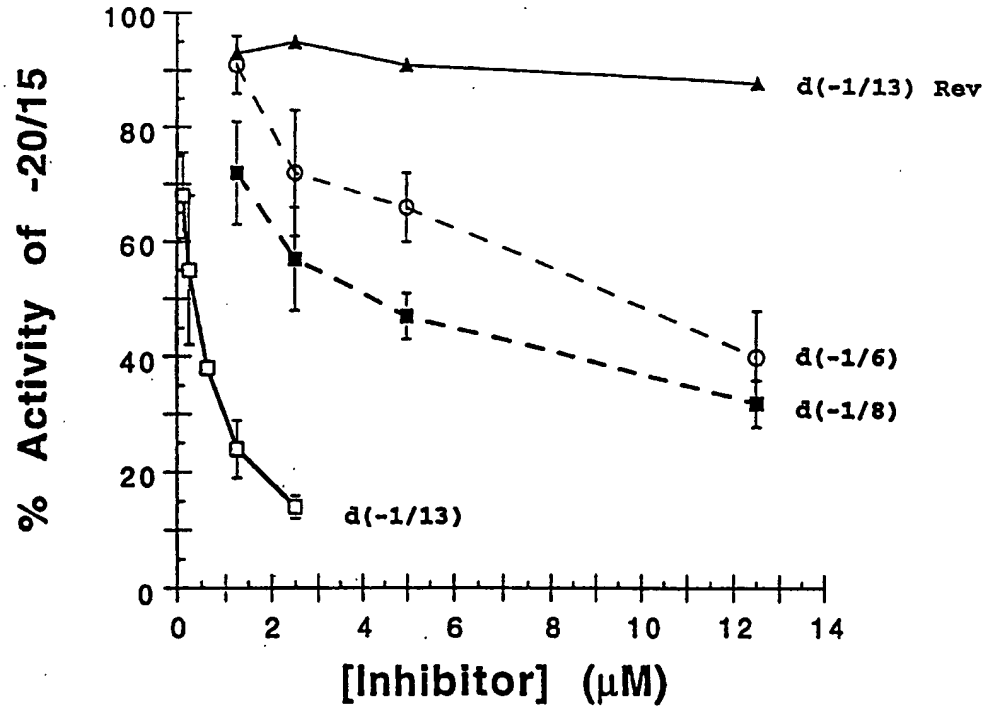


Figure 4. Minimal DNA Proscripts can inhibit viral RNA synthesis *in vitro*.

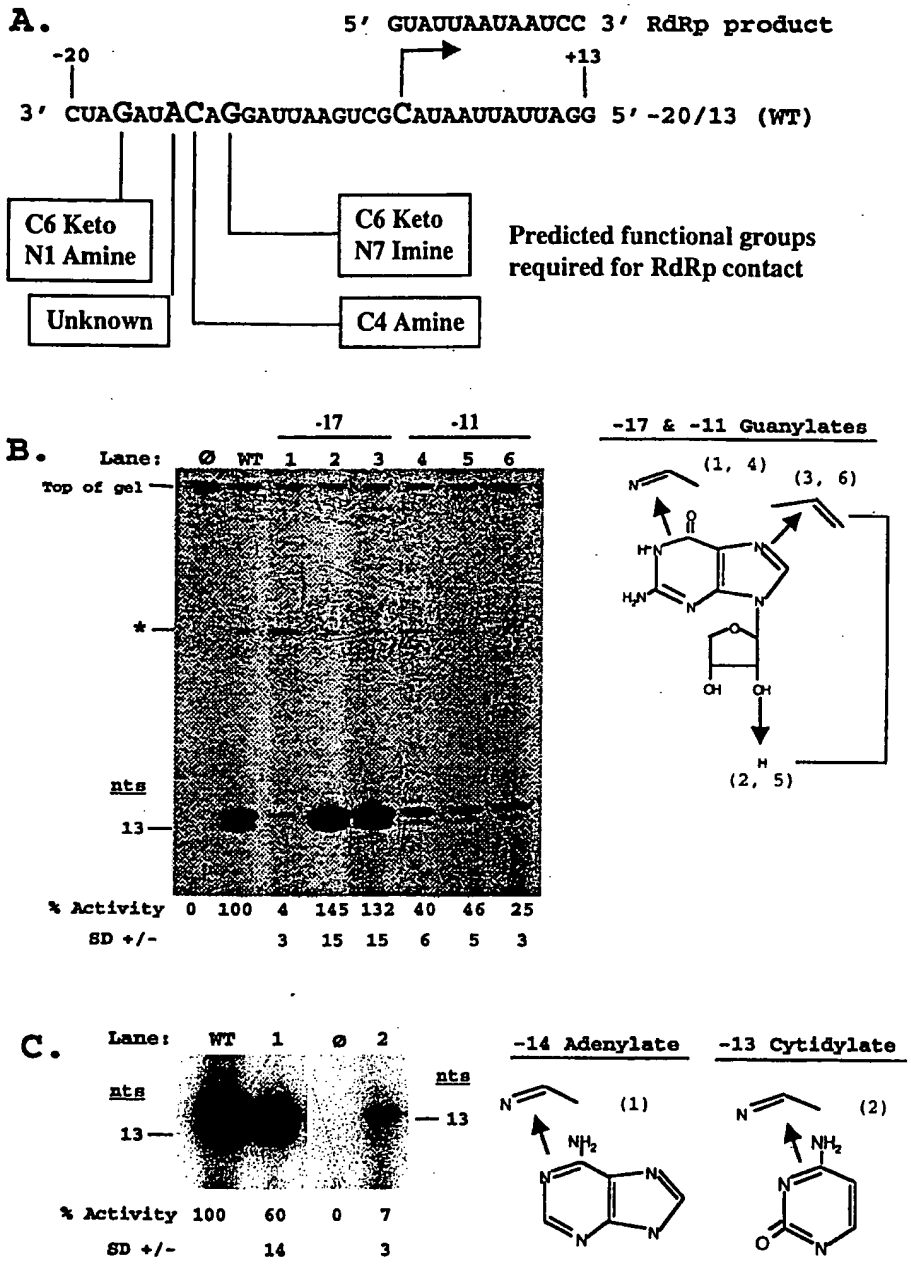


Figure 5. Functional moieties in the subgenomic promoter required for initiation of RNA synthesis

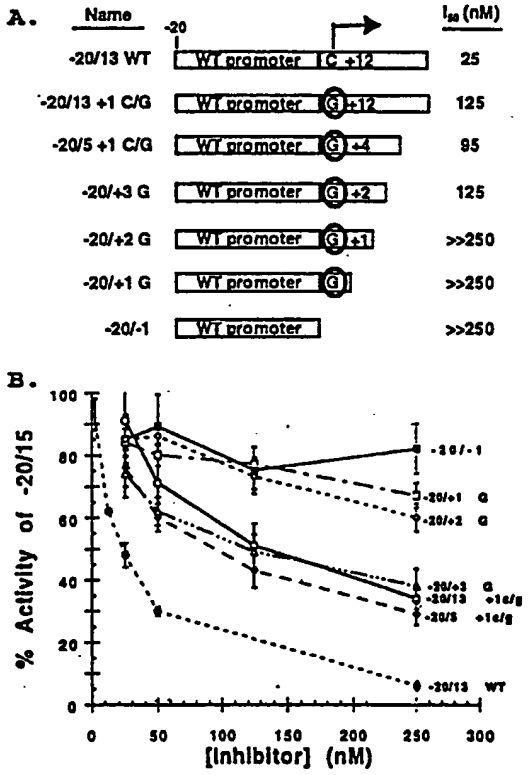


Figure 6.Template Requirements for Stable Interaction with RdRp.

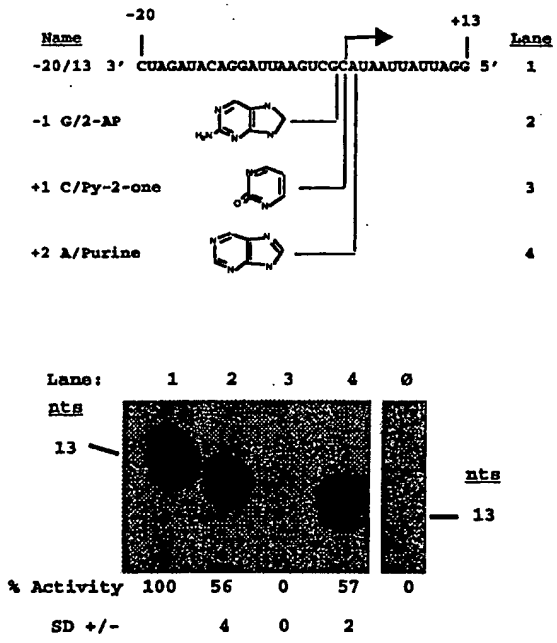


Figure 7. Recognition of the subgenomic initiation site

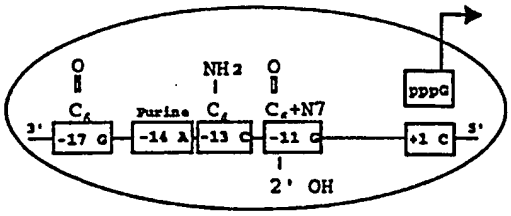


Figure 8. A Model for the Interaction Between the BMV RdRp and the Subgenomic Promoter Elements Needed to Initiate RNA Synthesis

Figure 9. Inhibition of BMV RNA Transcription Using Nucleic Acid Molecule

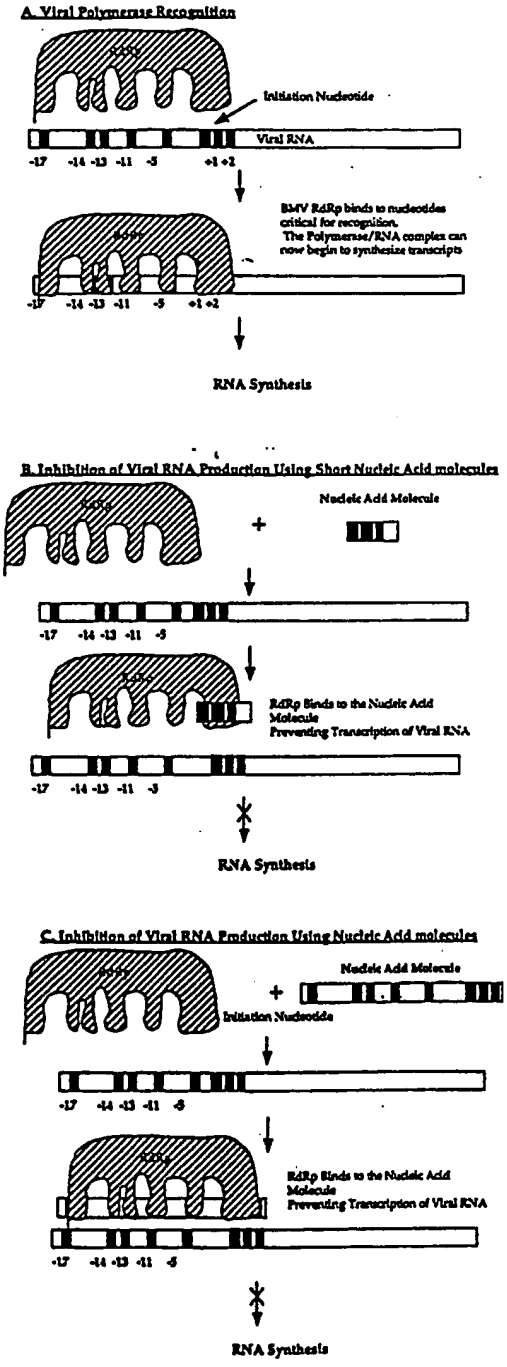


Figure 10. I_{50} Values of Selected Oligonucleotides

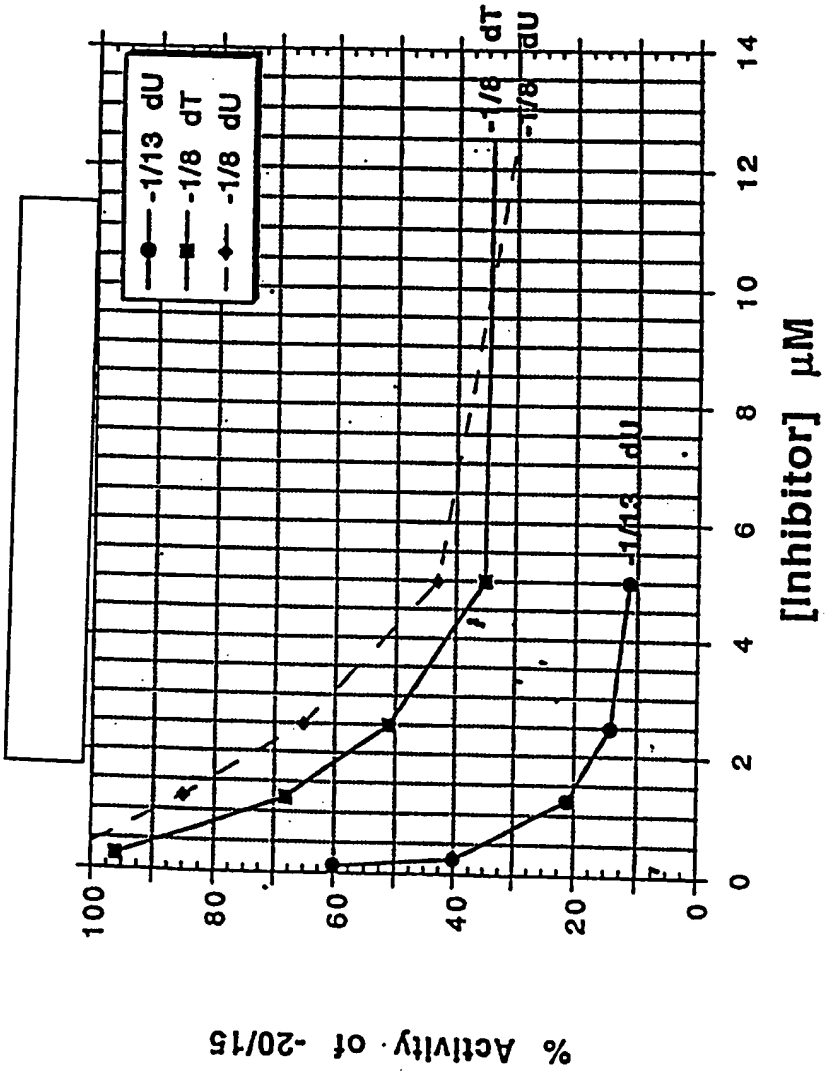
Proscript Name

-20/15 (WT) RNA

The amount of 15 nt product synthesized from this template which contains a WT subgenomic promtoer is measured in the presence of increasing amounts of the DNA inhibitors listed below.

	I_{50} (μM)	
-1/12 dU (contains deoxyuridines)	0.125	3' d(GCAUAAUUAUCAGG) 5'
-1/8 dU (contains deoxyuridines)	4.2	3' d(GCAUAAUGG) 5'
-1/8 dU (thymines replace uridines)	2.5	3' d(GCATAATGG) 5'

Figure 11. I50 Values for DNA Inhibitors



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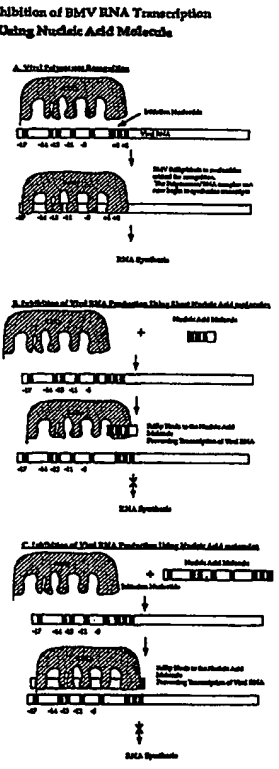
(57) Abstract

A linear single stranded nucleic acid molecule capable of specifically binding to a viral polymerase and inhibiting the activity of said viral polymerase.



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A. CLASSIFICATION OF SUBJECT MATTER
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Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 03668 A (ZENECA LTD ;ATABEKOV IOSSIF GRIGORIEVICH (RU); DOROKHOV YOURI LEON) 29 January 1998 (1998-01-29) abstract page 2 -page 3, line 3	1,2,4-8, 10-12, 18-22, 26-39
Y	claims	9,13-17, 21-25
Y	WO 97 26270 A (RIBOZYME PHARM INC) 24 July 1997 (1997-07-24) cited in the application abstract page 18, line 29 -page 22, line 2 page 87 -page 88; example 23	9,13-17

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
1 February 2000	07/02/2000

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PCT/US 99/16253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 10328 A (RIBOZYME PHARM INC ;DOWELANCO (US); ZWICK MICHAEL G (US); EDINGTON) 20 March 1997 (1997-03-20) claims 73-76	21-25
X	SIEGEL, R. ET AL.: "Sequence-specific recognition of a subgenomic RNA promoter by a viral RNA polymerase" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 94, October 1997 (1997-10), pages 11238-11243, XP002110076 ISSN: 0027-8424 cited in the application the whole document	1,3-8, 10,12, 18,34-38
X	ZACCOMER B ET AL: "TRANSGENIC PLANTS THAT EXPRESS GENES INCLUDING THE 3' UNTRANSLATED REGION OF THE TURNIP YELLOW MOSAIC VIRUS (TYMV) GENOME ARE PARTIALLY PROTECTED AGAINST TYMV INFECTION" GENE, vol. 136, 1 January 1993 (1993-01-01), pages 87-94, XP002047299 ISSN: 0378-1119 page 88, left-hand column, paragraph 1 page 91, paragraph D page 93, paragraph 2	1-5,8, 11,12, 18, 21-23, 26,27, 34,36-38
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A	the whole document	1-39
A	HUNTLEY C C ET AL: "MINUS SENSE TRANSCRIPTS OF BROME MOSAIC VIRUS RNA-3 INTERCISTRONIC REGION INTERFERE WITH VIRAL REPLICATION" VIROLOGY, vol. 192, 1993, pages 290-297, XP002047298 ISSN: 0042-6822 the whole document	1-39

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PCT/US 99/16253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	SIEGEL, R. ET AL.: "Moieties in an RNA promoter specifically recognized by a viral RNA-dependent RNA polymerase" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 95, September 1998 (1998-09), pages 11613-11618, XP002110078 ISSN: 0027-8424 the whole document	1-13, 18-38
P,X	ADKINS S ET AL: "Subgenomic RNA promoters dictate the mode of recognition by bromoviral RNA-dependent RNA polymerases." VIROLOGY, (1998 DEC 5) 252 (1) 1-8., XP002110079 the whole document	1-13, 18-38
E	WO 99 49031 A (KAO C CHENG ;SIEGEL ROBERT W (US); ADVANCED RES & TECH INST (US)) 30 September 1999 (1999-09-30) the whole document	1-39

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 16253

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 11 (as far as in vivo methods are concerned) is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ...nal Application No
PCT/US 99/16253

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9803668 A	29-01-1998	AU 3627997 A	10-02-1998
WO 9726270 A	24-07-1997	US 5728818 A	17-03-1998
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